Assuring the microbial safety and quality of Australian malt and barley

by

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Declaration

The thesis contains no material, which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge, contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

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Mandeep Kaur. March 2010

Mandeep Kaur
University of Tasmania

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Evans E. & Kaur M. (2009) Keeping sleepy yeast awake until bedtime. Understanding and avoiding PYF. *Brewer and Distiller International*, **5**, 38-40.

Kaur M., Stewart D.C., Sheehy M.C., Bowman J.P. & Evans D.E. (2009) Microbial diversity of barley malt grown under different environmental conditions, in diverse geographic locations (*In preparation to submit to Molecular Ecology*).

Kaur M., Stewart D.C., Sheehy M.C., Speers R.A., Lake J.C., Bowman J.P., & Evans D.E. (2009) Investigation of premature yeast flocculation using TRFLP and clone libraries (*In preparation to submit to Applied and Environmental Microbiology*).

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Kaur M., Evans D.E., Stewart D.C., Sheehy M.C., Speers R.A., Lake J.C. & Bowman J.P. (2009) Microbial TRFLP screening as a solution for premature yeast flocculation (PYF) assurance for malt. In: *32nd European Brewery Convention Congress*, Hamburg.

Kaur M., Evans D.E., Stewart D.C., Sheehy M.C., Speers R.A., Lake J.C. & Bowman J.P. (2009) Investigation of premature yeast flocculation (PYF) using TRFLP and clone libraries to develop a practical and reliable screening solution

for PYF malt. In: *American Society of Brewing Chemists Convention*, Tucson, Arizona.

Kaur M., Sheehy M.C., Stewart D.C., Speers R.A., Bowman J.P. & Evans D.E. (2008) The linking of microbial community analysis of barley and malt using terminal restriction fragment length polymorphism (TRFLP) with malt quality. In: *World Brewing Congress*, Honolulu, Hawaii.

Kaur M., Sheehy M.C., Stewart D.C., Bowman J.P. & Evans D.E. (2007) Characterization of microbial diversity in barley and malt using terminal restriction fragment length polymorphism (TRFLP). In: *13th Australian Barley Technical Symposium*, Perth, Western Australian.

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Chapter II has been published and reproduced in this thesis. Chapters III, IV and V have been written in the paper format and will be submitted subsequent to submission of sequence data to GenBank. The apparent repetition of text occurs so that each paper can stand alone.

List of abbreviations and acronyms

16S rRNA	16S rRNA is a part of the ribosomal RNA - a 1542 nucleotides long component of the small prokaryotic ribosomal subunit.
28S rRNA	structural RNA for the large component of eukaryotic cytoplasmic ribosome
ANOSIM	analysis of similarity
ANOVA	analysis of variance
AP	antimicrobial peptide
BioEdit	biological sequence alignment editor
BLAST	basic local alignment search tool
bp	base pair
Da	dalton
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DON	deoxynivalenol
EDTA	ethylenediaminetetraacetic acid
EFS1	early flocculation inducing substance number 1
EP	ethanol precipitate
G-50	gum 50
HMW	high molecular weight
HPLC	high pressure liquid chromatography
IACs	immunoaffinity columns
LOQ	limit of quantitation
LSD	least significant difference
m/z	mass to charge ratio
MDS plot	multidimensional scaling plot
MS	mass spectrophotometry
MVDISP	multivariate dispersion
NCBI	national center for biotechnology information
ns-LTPs	non - specific lipid transfer proteins
°Plato	degree plato, a measure of gravity

OTA	ochratoxin A
p value	level of significance
PAS I	periodic acid schiff I
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	photo – diode array
PYF	premature yeast flocculation
PYF +ve	premature yeast flocculation positive malt
PYF -ve	premature yeast flocculation negative malt
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SAB	south african breweries (Ltd., within South Africa)
SAB Miller	SAB Plc. worldwide
SIMPER	similarity percentage
SIMPROF	similarity profile
TRFLP	terminal restriction fragment length polymorphism
TRFs	terminal restriction fragments
UV	ultraviolet

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Abstract

Australia produces approximately 32% of the world's traded malting barley, ranking number one in the world for malting barley export. In the international export market, Australia has traditionally had a reputation for producing clean and bright barley/malt, which is presumably due to low microbial loads, resulting from dry conditions occurring during maturation and harvest.

The diverse microbial communities naturally colonizing barley grains greatly influence malt quality, and subsequently other products in the malt value chain, in particular beer. The objective of this thesis is to comprehend microbial diversity associated with barley and malt, thereby leading to a better understanding of cause of factors (such as microorganisms) that produce mycotoxins and cause premature yeast flocculation which impact brewing efficiency and beer quality.

Microbial fingerprinting of Australian malt and barley grown in different regions was benchmarked against malting barley grown internationally by using terminal restriction fragment length polymorphism (TRFLP) analysis. This approach was supported by cloning and sequencing techniques to assess microbial population composition. The TRFLP approach was considered the most appropriate because it is comparatively rapid, cost efficient and that microbial profiles from a large number of samples can be assessed. The TRFLP approach uses amplification of generic primers for the bacterial 16S rRNA gene and D1/D2 domain of the fungal 26/28S rRNA gene. Both qualitative and quantitative differences were observed in bacterial and fungal communities associated with malts produced in different geographical regions. The TRFLP and cloning approaches identified a greater diversity in yeast and filamentous fungi associated with barley malts than previously reported. Presumably this is the result of TRFLP being a culture independent approach, compared to traditional "wet plate" culture techniques which can bias towards the selective enrichment of fast growing microorganisms adapted to high substrate concentrations that can potentially represent a minor fraction of the resident microbial community.

Considerable differences in terms of bacterial and fungal populations were observed between Australian barley samples and their corresponding malts. The malts produced in different malt houses were dissimilar in terms of fungal community structure. Fungal clone libraries of different barley and malt samples demonstrated the absence of *Fusarium graminearum, Aspergillus* and *Penicillium* spp., the sources of deoxynivalenol (DON) and ochratoxin A (OTA). The absence of these mycotoxins in Australian malt was verified by testing malt samples for DON and OTA.

A new procedure for the regeneration of DON and OTA immunoaffinity columns was developed and used for detecting these mycotoxins in Australian barley and malts. This new regeneration method reduces the cost of screening for these commonly tested mycotoxins. None of the samples were found to contain detectable levels of either of DON or OTA. This outcome was attributed to the typically dry to hot climatic conditions in the Australian barley growing regions during the period from heading to maturity of the barley crop. In addition temperatures below 20°C at anthesis avoid the optimal conditions required for the infection of DON-producing *Fusarium graminearum* strains. Furthermore, the dry harvest conditions result in dry barley with moisture content (in this study, average 10.9% with a range of 8.7 – 12.4%) <13% for storage, which is well below the minimum moisture content (>14%) that is conducive for the growth of OTA-producing *Aspergillus* and *Penicillium* spp.

Premature yeast flocculation (PYF) is an intermittent brewing fermentation problem that results in incomplete wort fermentation, and is a significant problem for some breweries. The traditional approach to avoiding and solving this problem has been to detect PYF positive malts by using a small scale fermentation test. These fermentation tests are time consuming, expensive, sometimes inconsistent and difficult to transfer between testing laboratories. Research has also been directed at identifying the causal wort components (pectin/arabinoxylan or protein) of PYF. Neither of these approaches has been particularly successful over the past 40+ years. Consequently the problem was approached from a different and novel perspective. That was to use molecular finger printing as a step to identify the microbial taxa that cause PYF by comparing positive and negative malts using TRFLP, cloning and sequencing. A significant breakthrough has been made with this approach and a concept developed identifying substantial

differences between PYF positive and negative malts in their TRFLP (*Hae*III digestion) fungal fingerprints using the generic primers for the D1/D2 domain of the fungal 26/28S rRNA gene. This analysis indicates that more than one taxon of fungi are associated with PYF which perhaps indicates why previous researchers have had difficulty identifying the causal microbial taxa and causal agent/s.

Chapter I - Introduction and aims of the study

Barley (*Hordeum vulgare* L.) is a widely grown cereal crop in Australia, second in importance to wheat, planted on around 4 million hectares of arable cropping area. Australia contributes around 5 percent of the world's annual barley production. Possessing a small domestic market, Australian malting barley production is comparatively export focussed with approximately 80% of the total malting barley crop exported annually either as barley or as malt (Figure 1.1 A and B). To put this in global perspective Australia contributes around 32% of the world malting barley trade, ranking number one in the world for malting barley export (Figure 1.2). Australia also has an enviable reputation for producing a reliable supply of high quality, contamination free (microbes and chemicals) malting barley. The average Australian malting selection rate is the highest of the world's exporting nations around 38% of its national crop selected as malt (http://www.barleyaustralia.com.au/).

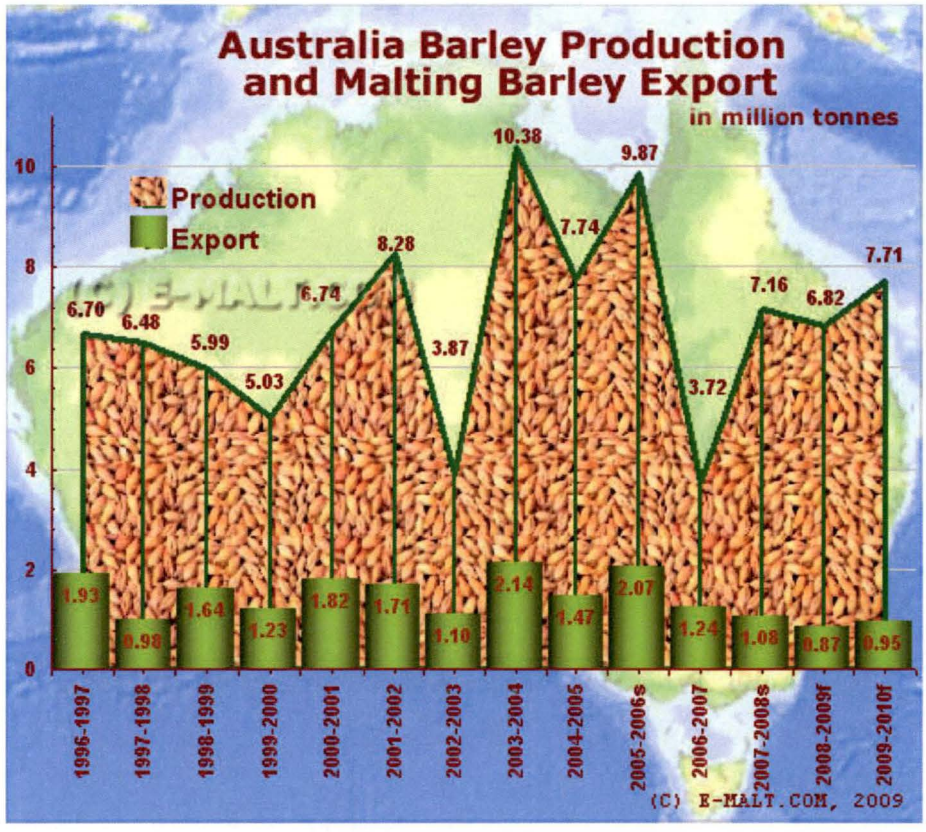


Figure 1.1 A: Australian barley malt production and export (http://www.e-malt.com/).

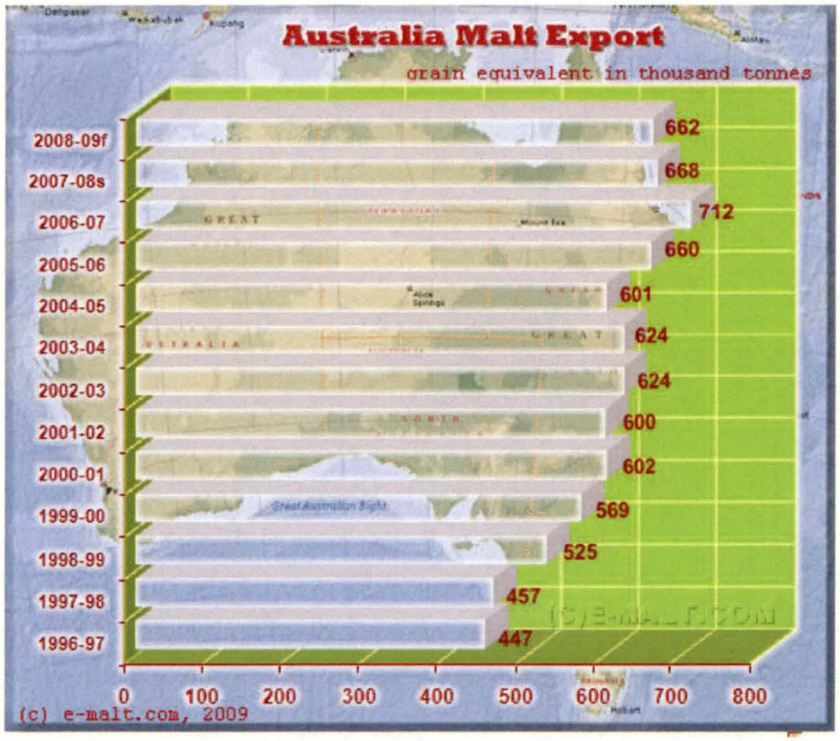


Figure 1.1 B: Australian barley malt export (http://www.e-malt.com/).

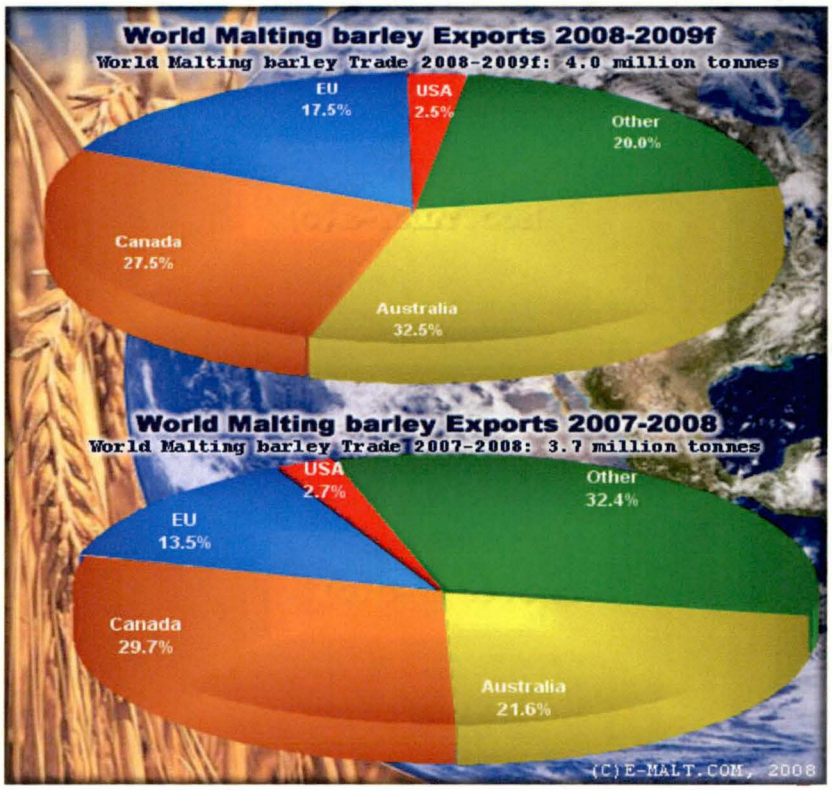


Figure 1.2: Australia's share in world malting barley export. (http://www.e-malt.com/)

1.1 Barley malting and brewing

Barley needs to be malted before brewing to enable the synthesis of enzymes that 1) soften the grain for easy milling; 2) assist wort separation by removing polymeric substances; 3) convert starch to fermentable sugars during mashing; 4) generate nutrients for yeast growth and maintenance; and, 5) eliminate undesirable proteins flowing into beer (Bamforth and Barclay 1993). Malting involves three main processes - steeping, germination and kilning. During steeping barley is immersed in water for 24 - 48 hr, where the grain imbibes water (usually to 42 – 46%), with two immersions generally of 8 hr each. Immersion is typically interrupted by 8 hr air-rest periods, in Australian malting. The wet barley grains are then allowed to germinate at 13 – 16 °C and 100% humidity for 3 - 6 days. Drying/kilning is the final stage of malting, whereby, green malt is gradually kilned starting at 50°C and finishing at 80 - 85°C over a 20 - 24 hr period. The purpose of kilning is to stop the growth of the green malt at the end of the germination and to produce a storage stable product containing satisfactory levels of active enzymes by reducing the moisture content to a level of 4 – 5%. The kilned malt is passed over screens to remove rootlets, coleoptiles, loose husk, dust and incomplete kernels. A pictorial overview is provided in Figure 1.3.

Screened (whole) malt is stored until the start of the brewing process. Storage can range from two weeks to a year or longer. The malt is then milled. The milled malt is mixed with water for mashing. Traditionally, mashing starts at a temperature of approximately 50°C (for 20 min) to allow the remaining action of thermolabile β – glucanase after which the temperature is increased to approximately 65°C (for 1 hr) for starch gelatinization and amylase enzyme complex activity. At the end of mashing wort (the liquid portion of mash) is recovered either by straining through the residual spent grains (lautering) or by filtering through "membranes" (mash filtration). The filtered wort is then boiled with hops or hop preparations (for 1 hr). In addition to removing protein precipitates, hop iso-α-acid isomerisation and unpleasant grainy characters, wort boiling aids in sterilization of the wort. Thereafter, the hopped wort is cooled, aerated, and pitched with yeast for fermentation (6 - 25°C). Fermentation is completed over 5 - 8 days during which the specific gravity drops to the desired

level and is followed by maturation or lagering during which the unpleasant butterscotch flavour (caused by diacetyl)

Figure 1.3: Overview of the malting process.

Procurement of barley from growers, storage and transportation.

Steeping – addition of water at 16 - 20°C separated by air rests to raise the grain moisture content from ~10-12% to 42 – 46%.

Germination – for 3 – 6 days at 13 – 16 °C, enzymatic breakdown of endosperm cell walls and proteins.

Kilning – drying of malt at progressively elevating temperatures (50 – 85 °C), while retaining much of the enzyme activity.

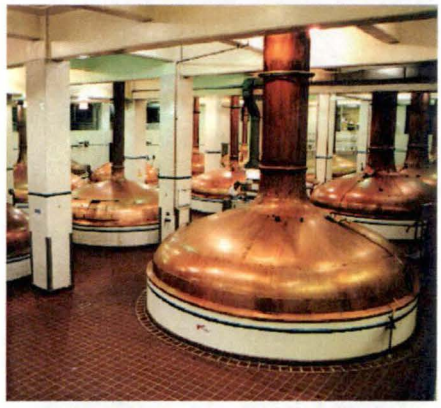
Screening, storage and transportation of malt.

and other green beer flavours have been metabolized by yeast. Beer is then conditioned (-1 to -2°C for several days or weeks), stabilized, pasteurized and filtered. The filtered beer is adjusted to the standard carbonation levels before packaging (Bamforth 2006). A pictorial overview is provided in Figure 1.4.

Figure 1.4: Overview of the brewing process.

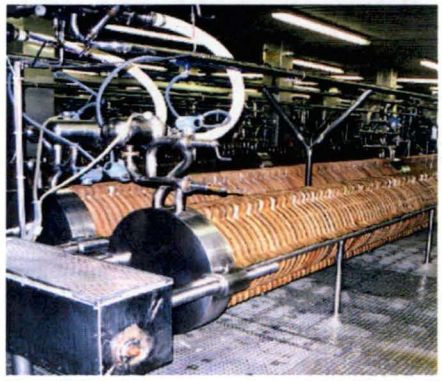
Milling – grinding of malts to generate grain particles to increase surface area for the action of mash water.

Mashing – mixing of grain particles and hot water (50 - 65°C, 1 – 2 hr), followed by wort separation.



Boiling – wort is boiled with hops or hop preparations (approx. 100°C, 1 hr). Followed by wort cooling, aeration and pitching.

Fermentation (6 - 25°C, 5 – 8 days), followed by maturation/conditioning for several days to weeks.



Beer filtration, then packaging.

1.2 Microbes, barley, malt and malting – a dynamic ecosystem

During barley growth, its storage and production of malt there are a number of critical points in the process that microbes have an opportunity to contaminate or colonise barley/malt.

1.2.1 Field microbiota

Microbial colonization of barley kernel occurs during all phases of kernel development including when the kernels are maturing in their natural positions in the ear. As the initial infection occurs in the field, the infesting microbes are called field microbiota. In general the field microbiota consists of bacteria, wild yeast and filamentous fungi that originate from both the air and soil. The type and extent of infestation varies according to variety, agronomic practices, climate and growing region (Etchevers *et al* 1977). Climate is believed to have the biggest role in determining the extent and composition of the microbiota (Beck *et al* 1991).

During kernel development in the field, the microbiota is numerically dominated by bacteria; as many as 10^8 cells per gram of barley have been isolated during late dough stage of grain development (Kotheimer and Christensen 1961). Bacterial species predominant on preharvest barley are *Erwinia herbicola* and *Xanthomonas campestris* (Flannigan 1996), but colonization by species belonging to *Pseudomonas*, *Micrococcus* and *Bacillus* genera also appear widespread even under dry harvest conditions (Haikara *et al* 1977).

Yeasts are the next most abundant microbes present on preharvest barley after bacteria. By harvest 50-85% of barley grains may be colonised by yeasts (Hill and Lacey 1983). The most frequently encountered yeasts are *Candida calenulate*, *Candida vini*, *Debaryomces hansenii*, *Hansenula polymorpha*, *Kloeceera apiculata*, *Rhodotorula muciloginosa*, *Sporobolomyces roseus* and *Trichosporon beigelii* (Petters *et al* 1988). Fungi are also omnipresent and the fungal genera commonly found on barley are *Alternaria*, *Acremonium*, *Aureobasidium*, *Cladosporium*, *Epicoccum*, *Fusarium* and *Verticillium* but there are important climatic and geographic influences (Flannigan 1996, Flannigan *et al* 1982, Haikara *et al* 1977, Schildbach 1989). In Australia, the typically dry environment

reduces canopy relative humidity and dry to hot harvest conditions presumably reduce the potential grain microbial load and grain moisture. As such Australia has a reputation for "bright" and clean barley with international grain buyers, presumably related to this low microbial load.

1.2.2 Storage microbiota

After harvest and before malting barley grains are stored to break inherent seed dormancy (Pyler & Thomas 2000). Considerable changes in microbial composition occur during this period depending upon the initial microbial composition, moisture content of the grain, storage time and conditions there within (Armolik *et al* 1956, Flannigan 1987). Haikara *et al* (1977) reported that barley with initial moisture content of 12.5% and stored for three months showed slight reduction in viable bacterial counts and the smallest reduction was in the numbers of *Bacillus* spp. because of greater durability of their spores. Grains at this stage are mostly dominated by Gram-positive bacteria represented by *Aureobacterium flavenscens*, *Bacillus* spp., *Brevibacterium linens*, *Corynebacterium* spp., *Clavibacterium iranicm*, *Microbacterium imperiale* and *Oerskovia xanthineolytica*, whereas Gram-negatives belong to *Erwinia herbicola*, *Pseudomonas fluorescens* and *Chromobacterium* sp. (Petters *et al* 1988).

Storage fungi are usually habitants of dust and air in the storage environment but can also be found in different equipment like harvesters and elevators (Flannigan 2003). Barley for storage in some areas requires drying to <14% moisture content which could potentially favours the growth of xerophilic fungi like *Aspergillus restrictus* and *Wallemia sebi*. Once the growth of microbes starts during storage their metabolic activity generates water and heat making path for the growth of less xerophilic and more thermotolerent fungi like *Eurotium*, *Penicillium*, *Thermoactinomyces*, *Micropolyspora*, *Rhizomucor* and *Thermomyces* spp. (Hill and Lacey 1983, Lacey *et al* 1980, Flannigan 2003). Elevated temperature (>30°C) at the beginning of storage, combined with high moisture content (15%) led to rapid invasion of Finnish barley with *Eurotium amstelodami* and *Eurotium rubrum* (Laitila *et al* 2003). A difference of <0.5% in grain moisture content can make a significant difference in the type of fungal species and their proliferation

during storage (Sauer et al 1992). However the distinction between field and storage fungal species is applicable generally to temperate climates, in warmer regions some species usually considered as storage fungi can already invade preharvest grain (Magan and Lacey 1984, Medina *et al* 2006).

1.2.3 Microbiota during malting

Apart from growth of microorganisms on the barley grain in the field and during storage, growth occurs during malting and subsequent storage of malt (Anderson *et al* 1967). The microbiota of finished malt is the result of initial microbial load of the incoming barley for malting, mutual interactions among different microbes during malting in relation to dynamic grain characteristics like moisture content and available nutrients and specific malting conditions used in different malting plants. Malting is considered to provide suitable conditions for microbial growth with regard to temperature, moisture and aeration (Flannigan 1996, Flannigan 2003, Noots *et al* 1999).

Steeping is a critical step in malting as populations of some microbes increase substantially during this period. Although some of the microbes are washed away along with steep water between air rests and before germination, the viable numbers increase substantially during the steeping period (Figure 1.5) and remain high throughout the germination period (Douglas and Flannigan 1988, Flannigan *et al* 1982, Petters *et al* 1988). An increase of up to 2-to 5 fold viable counts of filamentous fungi, 5-to 10 fold of yeasts and 50-to 100 fold of bacteria during steeping and germination has been reported by Follstad and Christensen (1962). Lactobacilli appears in substantial numbers, an increase of >×400 being proportionally much greater than for total viable bacteria during steeping (Petters *et al* 1988, O'Sullivan *et al* 1999, Sheneman and Hollenbeck 1960).

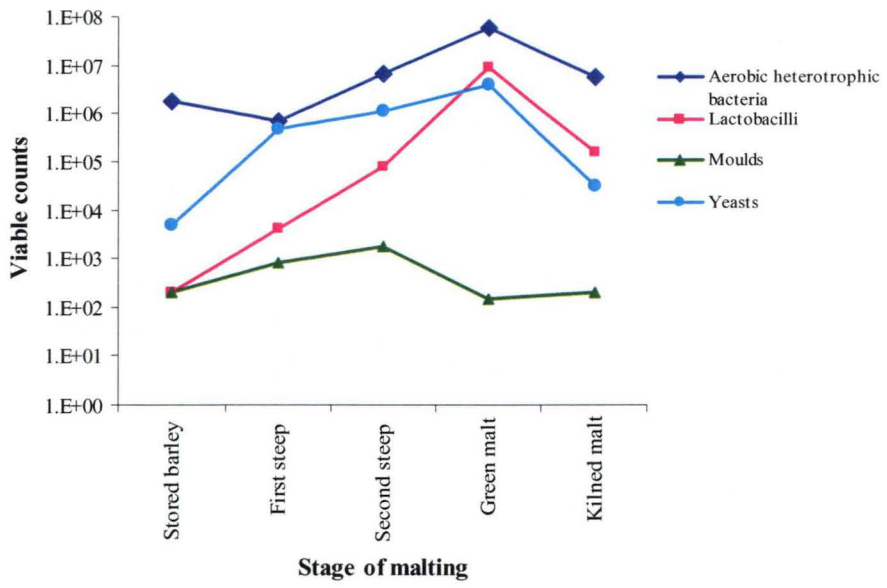


Figure 1.5: Viable counts of micro organisms associated with barley kernels during commercial production of sulphured malt (extracted from Petters *et al* 1988).

Further viable counts of bacteria and yeasts on green malt are much higher than on dry barley (Kotheimer and Christensen 1961). Yeasts and yeast like fungi reach the maximum level by the end of germination. Thermally tolerant yeasts, not detected in native barley, are able to multiply during the malting especially at the end of germination and at the beginning of kilning (Laitila *et al* 2006).

Studies have shown that *Fusarium* species grow at the beginning of malting followed by heat resistant fungi like *Aspergillus*, *Cladosporium* and *Penicillium*, while others like *Rhizopus* and *Mucor* species even reproduce rapidly at the early kilning temperatures (Douglas and Flannigan 1988, Haikara *et al* 1977, Schwarz *et al* 1995).The final 24 hour kilning stage dries the malt to around 4 - 5% moisture with hot air up to 85°C, markedly reducing the viable numbers of all the microbes, but the populations of all three groups i.e. bacteria, filamentous fungi and yeasts remain several times greater in kilned malt than in the original barley grains (Flannigan 2003, Follstad and Christensen 1962, Haikara *et al* 1977, Noots *et al* 1999, Petters *et al* 1988) (Figure 1.5). Thereafter, microbial growth and contamination of finished malt very much depends on the handling and storage operations adopted subsequently. High relative humidity accompanied by high

temperature and CO_2 concentration increases the chances of storage fungi proliferation (Flannigan 2003)

1.3 Microbial implications from grass to glass

1.3.1 Negative effects of microbes

The economically utilizable cereal harvest could be one third higher on a worldwide basis if it was possible to avoid losses (Schildbach 1989). A single fungal disease called Fusarium head blight or scab has the potential to completely destroy a potentially high yielding crop within a few weeks of harvest. The severe losses caused by scab in barley in South Korea in 1963 threatened some of the population with starvation (Vestal 1964). The damage from head scab is multi-fold: reduced yields, discoloured shrivelled grains, potential gushing in beer, contamination with mycotoxins, and reduction in seed quality resulting in difficulties in marketing, exporting, processing and feeding (McMullen *et al* 1997).

Microbial discolouration of the embryo and whole barley seeds is a problem world wide. Diseased kernels become dark brown to black on one or more sides or get a black appearance from the fungal biomass covering the kernel (Mathre 1997). Alternatively, the specific "black point" character may be more as a result of biochemical reactions than microbial load (Walker *et al* 2008). In the marketplace discoloured grain is discounted in value since cereal products made from it often have displeasing colour and odour characteristics. Particularly barley is unfit for malt production when infected with microbes as the germination of such kernels is decreased (Basson *et al* 1990, Briggs 2004, Doran and Briggs 1993, Saric *et al* 1997, van Campenhout *et al* 1998). Several fungal and bacterial genera have been reported to exert the potential to kill or reduce embryo vigour (Hudec 2007, Laitila *et al* 2007, Schwarz *et al* 2001). Poor germination of weathered grains may be due to microbial competition for oxygen and or due to the formation of physical barriers like biofilms (Bishop 1944, Laitila *et al* 2007, Lynch and Prynn 1977, Morris and Monier 2003). In addition, water sensitivity, the inability of the kernel to germinate under conditions which reduce the rate of

oxygen entry, have been reported to be due to severe microbial infestation (Gaber and Roberts 1969).

Pronounced differences in barley kernel composition and barley and malt wort quality have been reported to result from microbial contamination. Several species of *Fusarium*, *Aspergillus*, *Mucor*, *Rhizopus* and *Pseudomonas* are potential sources of proteinase, β-glucanase and xylanase enzymes which cause undesirable increase in wort free amino nitrogen, soluble nitrogen and wort colour, and decrease in β-glucan (Gyllang *et al* 1977, Prentice and Sloey 1960, Sarlin *et al* 2005, Schwarz *et al* 2001, Schwarz *et al* 2002). Changes in wort colour by *Fusarium* spp. may be caused by pigments associated with the fungal mycelium or by an increased amount of melanoidins as a result of improved malt modification by microbial enzymes (Gjertsen *et al* 1965, Sloey and Prentice 1962). The decrease in β-glucan results in lower wort viscosity, which has been negatively correlated to beer foam quality (Evans *et al* 1999). Poor filterability of wort and beer has been related to extracellular polysaccharides produced by yeasts and bacteria (Anderson 1993, Haikara and Home 1991, Kreisz *et al* 2001). Recently, Zhang *et al* (2009) concluded that wort turbidity and lautering could be specifically attributed to extracellular polysaccharides from *Aspergillus flavus*.

Process dependent organic acid profile and the final concentration of these acids in malt are important factors of variation in wort and beer pH. Haikara and Home (1991) observed sour and acidic off flavour in mash and low wort pH was the result of intensive growth of lactic acid bacteria during malting of split barely grains. Microbial contaminated barley/malt can result in transfer of fine particulate matter in beer making it hazy (Etchevers *et al* 1977).

One of the well known effects of the microbiota on barley and malt is that of gushing. This is a phenomenon in which beer spontaneously, without agitation, overfoams, sometimes vigorously, out from the package immediately after package opening (Gjertsen 1967). Although initially entertaining, consumers are not pleased when most of the beer erupts from the package and in severe cases it can literally hit the roof! Negative brand image from such an effect results in

significant economic losses for the maltsters and brewers. Often correlated to a humid and rainy flowering and harvesting periods, which promote fungal growth on barley kernels (Gjertsen *et al* 1963) gushing factors can also be produced by fungi during storage or malting. Primary gushing is commonly attributed to *Fusarium* spp. (Haikara 1980, Hippeli and Hecht 2009, Laitila *et al* 2007, Prentice and Sloey 1960, Schwarz *et al* 1996, Sloey and Prentice 1962) but other genera like *Penicillium*, *Nigrospora*, *Stemphylium*, *Alternaria* and *Aspergillus* are also reported to produce gushing factors (Amaha *et al* 1973, Gyllang *et al* 1977, Kitabatake and Amaha 1974, Yoshida *et al* 1975). One hypothesis is that microbial infection results in accumulation of non-specific lipid transfer protein (ns-LTP1), and when ns-LTP1 content reaches a certain threshold primary gushing is induced (Hippeli and Hecht 2009). However, gushing is a very complex phenomenon and can partly be explained by the production of hydrophobins by fungi either in the field or during malting (Hippeli and Elstner 2002, Kleemola *et al* 2001, Sarlin *et al* 2005).

Spores of certain fungi and bacterial actinomycetes present in grain dust are potent source of allergens, thus posing a continuous health risk to people working in the grain industry. Allergic reaction to spores of *Aspergillus clavatus* and *Aspergillus niger* are reported to cause malt worker's lung and brewer's asthma in workers (Grant *et al* 1976, Heaney *et al* 1997).

Mycotoxins

In addition to allergens, some fungal species associated with barley grains are capable of producing mycotoxins that are harmful to animal and human health. Mycotoxins are secondary metabolites produced by fungi and are considered non-essential for the growth of fungi as compared to primary metabolites like amino acids and nucleic acids (Bilgrami and Choudhary 1998). Mycotoxins result from fungal infestation of barley in the field or during storage and their levels may be increased during the malting process. Several genera of moulds are capable of producing mycotoxins (Table 1.1). The most important are: *Aspergillus*, *Fusarium* and *Penicillium* (Sweeny and Dobson 1998).

Table 1.1: Important mycotoxins detected in barley and malt and producing microorganisms (adapted from Noots *et al* 1999).

Fungal species	Mycotoxin
Storage fungi	
Aspergillus flavus	Aflatoxin B1
Penicillium spp.	Citrinin
Aspergillus ochraceus, Penicillium verrucosum, Penicillium viridicatum	Ochratoxin A (OTA)
Field fungi	
Fusarium culmorum, Fusarium graminearum	Deoxynivalenol (DON), Zearaleneone
Fusarium culmorum, Fusarium graminearum, Fusarium poae, Fusarium sporotrichioides	Diacetoxyscirpenol
Fusarium poae, Fusarium sporotrichioides	T-1, T-2 toxin

In malting and brewing environments typically ochratoxin A (OTA) and the type B trichothecene, deoxynivalenol (DON), are screened for. Thus DON and OTA are indictors for field and storage fungal contamination, respectively. Deoxynivalenol or vomitoxin has been reported to cause nausea, vomiting, abdominal pain, diarrhoea, dizziness and headache in humans. Whereas OTA, has been shown to be nephrotoxic, immunosuppressive, carcinogenic, and teratogenic to mammals (Peraica *et al* 1999). Along with the food safety issues associated with mycotoxins the implications these fungal secondary metabolites pose during malting and brewing processes are of great concern to the malting and brewing industry. The inclusion of 50 µg/ml of DON in a cell culture medium resulted in up to a 15% reduction in cell number, dry mass and total protein during growth of *Saccharomyces cerevisiae* yeast thus reducing brewery performance (Whitehead and Flannigan 1989). Boiera *et al* (1999) also reported that DON was inhibitory for both top (ale) and bottom (lager) fermenting yeasts. Schapira *et al* (1989) reported that DON along with other trichothecenes affect the development of rootlets, coleoptile and enzyme synthesis, during the malting of barley.

To date there have been no published report of mycotoxins occurring in Australian barley and malt. This is mainly due to Australia's climate which is relatively cool (below 20°C) around anthesis reducing the risk of *Fusarium*

graminearum or *Fusarium culmorum* (major DON producing species) infection and thus DON production (Prom *et al* 1999, Schwarz *et al* 1995, Webley and Jackson 1998, Webley *et al* 1997). Further, dry harvest conditions result in dry barley with a moisture content <13% for storage which is well below the minimum moisture content (>14%) that is conducive for the growth of OTA producing fungi (*Aspergillus* and *Penicillium* spp.) thus OTA production (Elmholt and Rasmussen 2005, Leoni and Furlani 2001, Magan and Aldred 2005). However, screening for quality assurance purposes is still required, because in the unlikely event of barley infection by either field or storage fungi such as *Fusarium, Aspergillus* and other fungal species, harmful mycotoxins could potentially accumulate. Brewers' quality control protocols understandably require that malting barley is free of mycotoxins such as DON and OTA.

Premature yeast flocculation (PYF)

Premature yeast flocculation is an intermittent issue in the brewing industry that results in incomplete yeast utilisation of fermentable sugars in the wort, resulting in out of specification beer and disrupted brewing production schedules, leading to significant economic losses for the effected brewer (Axcell *et al* 2000). Previous investigations have identified that PYF is associated with certain batches of malt produced due to unfavourable and ill-defined interactions with barley microbes (van Nierop *et al* 2006). As such, as the awareness of PYF increases it is becoming an increasingly important quality issue especially for malt exports to large multinational brewers who are setting quality targets for this parameter (Griggs *et al* 2008, Jibiki *et al* 2006).

The incomplete understanding of PYF originates from the fundamental question, what actually is PYF? There are two different although related definitions of PYF. One group defines acute PYF as early flocculation during primary fermentation to produce beer with unacceptably high levels of residual fermentable sugars (gravity) (Figure 1.6A). As this premature yeast flocculation occurs during primary fermentation, this type of PYF was called primary PYF. While, the other school of thought recognises a more subtle, chronic PYF where the cell count in suspension during maturation/secondary fermentation is at a sub-optimal level so

that the removal of undesirable flavour components such as diacetyl (butterscotch flavour) is incomplete (Figure 1.6B) (Lake and Speers 2008b, van Nierop *et al* 2004). As this type of PYF occurs during secondary fermentation, on the same line as primary PYF this type can be referred as secondary PYF.

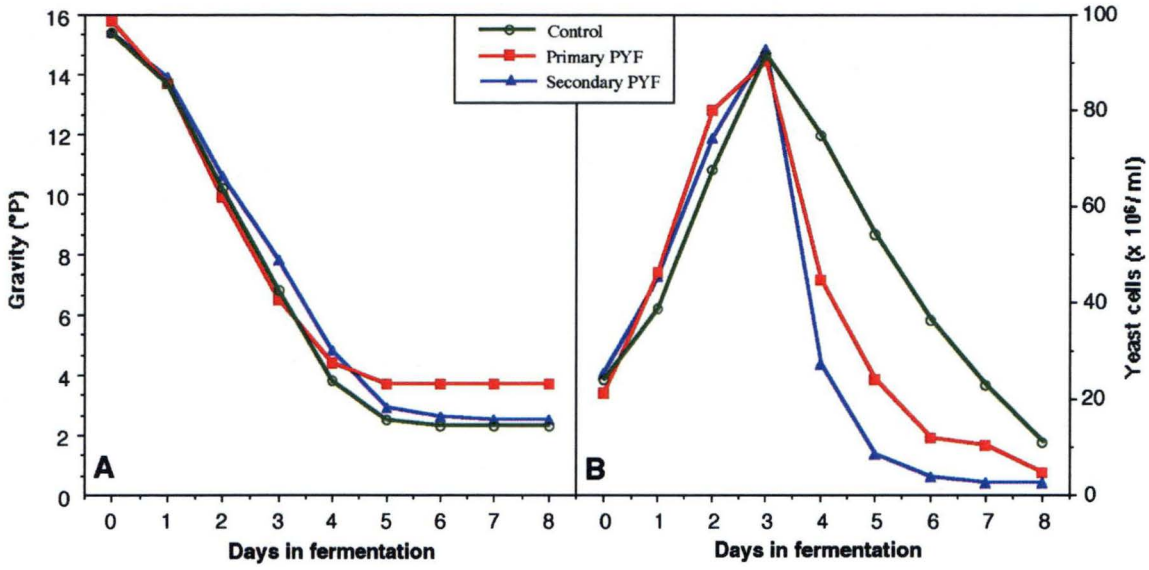


Figure 1.6: The symptoms of PYF observed by brewers. A. Wort not fermented, out to the desired gravity. B. Low green beer yeast cell concentrations leading to insufficient yeast numbers for beer maturation/lagering (after van Nierop 2004).

Several substances in the wort derived from malt, particularly the husk, have been implicated in inducing PYF (Axcell *et al* 2000, Fujii and Horie 1975, Fujino and Yoshida 1976, Gorjanović *et al* 2004, Herrera and Axcell 1989, Herrera and Axcell 1991a, Herrera and Axcell 1991b, Koizumi *et al* 2008, Lake and Speers 2008a, Morimoto *et al* 1975, van Nierop *et al* 2004). Table 1.2 lists the putative causal substance/substances that have been identified as either a carbohydrate, protein or their combination (i.e. glycoprotein). Similarly the size of the PYF factor has remained ill defined (>100,000 Da - <5000 Da) because of confounding interactions between brewing raw materials and the brewing processes. There is also possibility that PYF might be caused by more than one compounds acting individually or in a synergistic manner (Lake and Speers 2008b, van Nierop 2004, Wood *et al* 2005).

Table 1.2: Factors indicated or associated with premature yeast flocculation in literature (adopted and extended from Lake and Speers 2008).

Factor description	Size	Effect	Citation
Barmigen – humic acid-like substance containing ash (11%), carbon (47.56%), hydrogen (4.92%), and nitrogen (3.14%)	HMW	Caused flocculation in buffered solution	Kudo 1958, Kudo and Kijima 1960
Treberin – gum-based polysaccharide containing glucose, xylose, and arabinose	-	Associated with PYF	Kudo 1959
EFS1 – glycoprotein with a negative charge; sugar components: galactose> arabinose> glucose> xylose> mannose and inorganic phosphorous> organic phosphorous> polyphenol	HMW	Associated with PYF	Fujii and Horie 1975
EP – a mixture of arabinoxylan, α-glucan, and glycoprotein consisting of two polysaccharides	HMW	Associated with PYF	Morimoto *et al* 1975
G-50 – gum-based polysaccharide containing arabinose (44%), xylose (34%), glucose (15%), and an unidentified component (7%)	HMW	Associated with PYF	Morimoto *et al* 1975
Peptide high in glutamic and aspartic acids	<10,000 Da	Not specifically associated with PYF	Stewart *et al* 1976
FB – glycoprotein composed mainly of glucose, galactose, and mannose with traces of xylose and arabinose; minor amount of nitrogenous constituents	-	Associated with PYF	Fujino and Yoshida 1976
FA – glycoprotein composed mainly of mannose, xylose, arabinose with traces of galactose, and glucose; nitrogen with uronic and ferulic acid also detected	-	Associated with slight PYF	Fujino and Yoshida 1976
Barley lectin	20,700 Da	Not specifically associated with PYF	Herrera and Axcell 1989
PAS I – gum-based polysaccharide composed of arabinose (27%), xylose (17%), mannose (17%), galactose (16%), rhamnose (14%), and glucose (12%), with an acidic sugar component	>100,000 Da	Associated with PYF	Herrera and Axcell 1991a, Herrera and Axcell 1991b
Lipid transfer protein	≤10,000, 16,000 Da	Not specifically associated with PYF	Axcell *et al* 2000, Evans *et al* 2002, van Nierop 2005, Gorjanović *et al* 2004
Arabinoxylan products of husk degradation by endo-xylanase and *Aspergillus niger*	HMW	Associated with PYF	van Nierop *et al* 2004
Complex polysaccharide containing arabinose (31%), xylose (21%), galactose (12%), glucose (9%), rhamnose (9%), and mannose (3%)	≈40,000Da	Associated with PYF	Koizumi *et al* 2004
High molecular weight polysaccharide PYF factor –with ferulic acid as an active ingredient	≥100,000 Da	Associated with PYF	Lake and Speers 2008a
Fraction III and V – polysaccharide composed of arabinose, galactose, xylose, rhamnose, galacturonic acid, mannose, glucose, and glucuronic acid	<40,000 Da	Associated with PYF	Koizumi *et al* 2008
Sanzyme 1000 enzyme digestion product of fractions III and V	<5000 Da	Associated with PYF	Koizumi *et al* 2008

The identity of the PYF factor/s has yet to be elucidated, the biochemical background for the formation and mode of action of PYF is yet to be fully understood. However, it is most likely that PYF is produced as a result of microbial contamination of barley grains in the field, with further proliferation during malting especially under favourable steeping conditions (Armstrong and Bendiak 2007, Axcell *et al* 1986, Axcell *et al* 2000, Blechová *et al* 2005, Griggs *et al* 2008, Sasaki *et al* 2008, van Nierop *et al* 2004, Yoshida *et al* 1979). The action of microbes on barley malt in inducing PYF is proposed to be two fold (Figures 1.7 and 1.8); firstly microbes associated with the barley husk secrete enzymes that breakdown the cell wall of grains into assimilable nutrients for the microbes (van Nierop *et al* 2004). Degradation products such as acidic high molecular weight (HMW) polysaccharides in combination with Ca^{2+} cross-link the lectin-like proteins on the yeast cell surface forming flocs of yeast cells. Secondly and additionally microbial stress triggers an anti-pathogenesis immunological type response from the barley grains, during grain maturation and malting, resulting in the accumulation of plant defensins, antimicrobial peptides such as non-specific lipid transfer proteins (ns LTPs). These plant defensins are suggested to impair yeast cell metabolism, respiration and cell membrane integrity causing irreversible cell injuries (Axcell *et al* 2000, Gorjanović *et al* 2004, Lake 2008, Lake *et al* 2008, van Nierop *et al* 2004, van Nierop 2005, Wood *et al* 2005). Recently, van Nierop *et al* (2008) developed an optimised anti-yeast assay to differentiate barley malt batches according to their antimicrobial activity towards a specific lager brewing yeast strain. The assay revealed that malts associated with PYF had high anti-yeast activity suggesting microbial association with PYF.

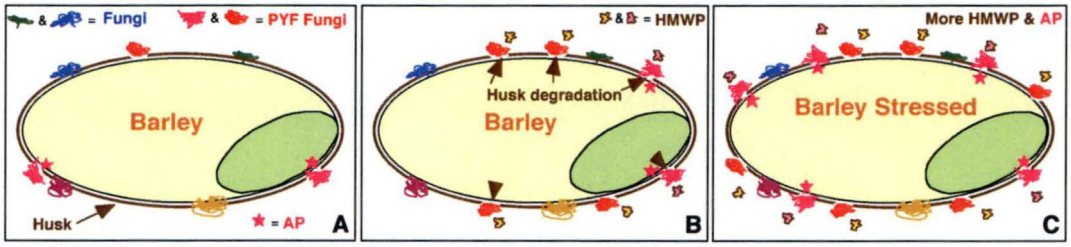


Figure 1.7: Proposed mechanism of premature yeast flocculation factor(s) generated from barley husk by fungi. A: Normal infestation by fungi: B: fungal enzymatic degradation of the husk with PYF causing fungi: and C: Heavy fungal infestation and production of more antimicrobial peptides (AP) by barley, HMWP= high molecular weight polysaccharides (after van Nierop *et al* 2004).

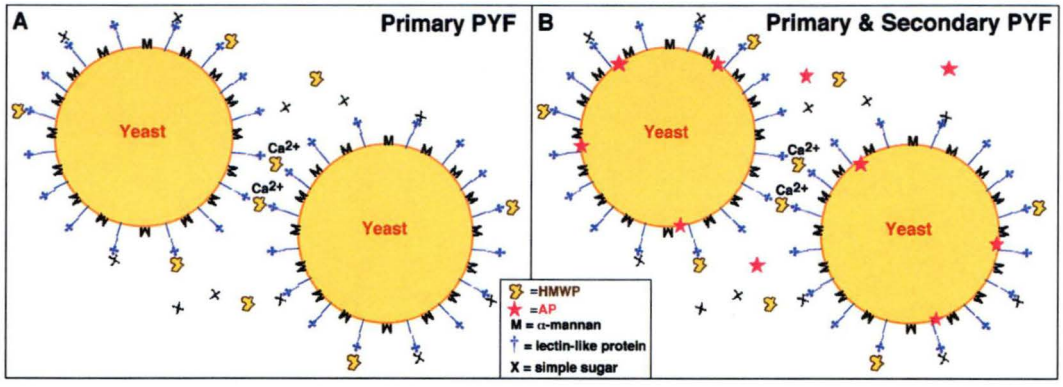


Figure 1.8: Proposed mechanism of premature yeast flocculation by A: high-molecular weight polysaccharides (HMWP) causing primary PYF only: and B: Primary and secondary PYF caused by HMWP in association with antimicrobial peptides (AP) (after van Nierop *et al* 2004).

To date no physical or chemical analysis methods have been developed to routinely detect the presence of the PYF factors in malt or barley. Consequently, the brewing industry relies on fermentation assays (Table 1.3) that are expensive, time consuming, and inconsistent (Lake and Speers 2008b). A complicating factor is that different strains of yeast and their metabolic status (i.e. brewing cycle age) determine their propensity to prematurely flocculate (van Nierop *et al* 2006). The differing yeast strains susceptibility to PYF explains the variable degree of concern between brewing groups towards the PYF problem and presents difficulties in finding a universal test for PYF. As the flavour of the brewers beer brands are highly dependant on the yeast strain, changing yeast strain is not an

option (van Nierop *et al* 2006). Some success has been reported in the downsizing, and increasing the speed of these assays although real problems remain in the transferability and reproducibility of these assays between testing laboratories. This is possibly explained by the importance of the strain of yeast used and its metabolic state. The lack of consensus with respect to selecting a universal standard assay for PYF is a substantial problem, which makes comparison of research reports on PYF difficult, as one assay may deem a particular malt batch PYF positive, while another assay may not. In addition, a positive result in the test may not necessarily translate into a problem in the brewery or vice versa.

Table 1.3: Some of the fermentation assays to test PYF potential of barley/malt.

Test	Description	Citation
Kirin test	7 day fermentation to compare turbidity with control - absorbance measured at 800nm	Fujino and Yoshida 1976
Improved Kirin test	8 day fermentation to compare turbidity with control - absorbance measured at 800nm	Inagaki *et al* 1994
Nakamura barley PYF test	4 day test, 50g barley mashing coupled with enzymes followed by 48hr fermentation and 800nm absorbance measurement	Nakamura *et al* 1997
SAB-Miller test	Small scale fermentation in Kadena-Danish like tube to measure excess flocculation	van Nierop *et al* 2004
Rapid Kirin test	5g malt, 50g barley extract and ethanol precipitate. Approx 3 hr, absorbance 600 ratio with control	Koizumi and Ogawa 2005
Asahi test	48 hr fermentation with absorbance 600 ratio with control	Jibiki *et al* 2006
Lake test	<72 hr 15ml test tube fermentation at 21°C with 4% added glucose, measure turbidity at A600, °Plato and shear rate	Lake *et al* 2008
Anti yeast assay	Micro titre plate scale, absorbance 600 measurement	van Nierop *et al* 2008

Despite the consensus of opinion being that PYF stems from microbial contamination of barley/malt, relatively little work has been reported on linking specific barley or malt microbial taxa with PYF malt. Table 1.4 lists a number of different taxa of microbes associated with the occurrence of PYF. As a number of different microbial taxa have been identified to be associated with PYF, it appears highly likely that the occurrence of PYF is dependent on the interactions between microbial taxa. Furthermore, most of the reported work has been directed towards fungal studies with little effort being devoted to the other and main microbial "component," bacteria.

Table 1.4: Microbes indicated or associated with premature yeast flocculation in literature.

Fungi/Bacteria	Effect	Citation
Lactobacillus fermentum	Not specifically associated with PYF	Zarattini *et al* 1993
Aspergillus aculeatus, *Aspergillus ficuum*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus terreus*, and *Fusarium culmorum*	Associated with PYF	van Nierop *et al* 2004
Unspecified fungi	Associated with PYF	van Nierop *et al* 2004
Fusarium culmorum and *Fusarium graminearum*	Associated with PYF	Blechová *et al* 2005
Aspergillus fumigatus, Fusarium sp., and *Rhizopus* sp.	Associated with PYF	Yang *et al* 2007
Aspergillus candidus, *Cladosporium cladosporiodes*, *Penicillium cyclopium*, *Penicillium melanconidium,* and *Penicillium viridicatum*	Associated with PYF	Sasaki *et al* 2008

1.3.2 Positive effects of microbes

There is an increasing demand for organic products from producers and consumers all over the world. Maltsters and brewers are also focusing on the possibility of using malting barley grown without the use of synthetic pesticides for beer production. Naturally occurring substances are also becoming important sources of antifungal agents. They can be developed either as products *per se* or used as starting points for synthesis of new compounds. Pyrrolnitrin produced by *Pseudomonas pyrrocinita* and strobilurus A produced by *Strobilurus tenacellus* are two such fungicides which have been used commercially (Gullino *et al* 2000). To date most research into barley and malt microbes has focussed on the undesirable microbial interactions. However, as Laitila (2008) recently pointed out, on the whole, the impact of microbes on malt quality is "*more good than bad*".

Microbes originating from barley, malt or brewery environments offer a potential alternative as natural food grade biocontrol agents. Yeast species, such as *Geotrichum candidum*, *Pichia anomala*, the filamentous fungi *Rhizopus* spp. and several lactic acid bacteria have successfully been applied as starter cultures in malting (Boivin and Malanda 1996, 1997, Dufait and Coppen 2002, Dziuba and Foszczyńska 2001, Laitila 2007, Laitila *et al* 1999, O'Mahony *et al* 2000). These starter cultures not only inhibit the undesirable microbiota but also enhance malt modification by producing xylanase, β glucanase and proteases enzymes and result in beneficial effects like lower wort β glucan, wort viscosity, and better wort filtration further down the beer processing chain (Boivin and Malanda 1997, Haikara and Laitila 1995, Laitila *et al* 2002, Laitila *et al* 2006, Lowe *et al* 2005). *Pichia anomala* VTT C-04565 (C565) has been observed to restrict *Fusarium* growth and hydrophobin production during malting and prevented beer gushing. Further, addition of *Pichia anomala* C565 seemed to retard wort filtration, but the filtration performance was recovered when yeast culture was combined with *Lactobacillus plantarum* VTT E-78076 (Laitila *et al* 2007, Laitila 2007). Strains of lactic acid bacteria which produce bacteriocins in addition to organic acids appear to be a promising approach for biopreservation of foods (Hartnett *et al* 2002, Todorov *et al* 2003, Vaughan *et al* 2001).

A significant loss of original barley weight occurs during the malting process due to the removal of rootlets. A reduction of up to 50% in these malting losses is achievable if malt is treated with *Lactobacillus plantarum* 15GR starter culture, while keeping good malt quality at the same time (Schehl *et al* 2007).

1.4 Study context and aims

As discussed above, the barley grain carries with it a community of micro-organisms referred to as its microbiota. An extensive listing of the microbial species and genera has been displayed in literature and it is suggested that the microbiota of different barleys are similar to each other and to other cereals, dominated by a relatively small number of species though a range of microorganisms has been isolated. Most of these studies have primarily used grain sourced from the relatively humid, wet harvest climates of Northern Europe and North America. Further, given the microbial presence on barley grain and the favourable conditions for their multiplication during malting, there has been relatively little study of their impact on the assessment of malt quality characteristics such as presence of mycotoxins and PYF factors.

To date relatively few investigations have been reported for the microbiology of Australian barley or malt. Typically Australian barley is grown under less humid conditions and harvested during the dry periods of the year. Further, most investigations have been centred on wheat, particularly on the incidence of *Fusarium graminearum* head blight that is climatically restricted to North eastern Australian cropping zone (Backhouse and Burgess 2002), with the last major outbreak in the mid 1980s (Blaney *et al* 1987, Burgess *et al* 1987). Surveys of mycotoxins, again have mainly centred around wheat (Blaney *et al* 1987) with Webley and Jackson (1998) concluding that in comparison to North America and Europe, Australia had a low risk of containing DON and related mycotoxins. It is therefore clear that a comprehensive examination of the microbiota of Australian barley and malt and its effect on malt quality is long overdue. Keeping these points in mind, the present study was conducted with the overall aim of determining the typical microbial composition and load for Australian malt and barley grown in different environments and areas compared to malting barley

grown internationally. Within this overall aim the more specific objectives are as follow:

1. Develop and apply an improved method for measurement of key mycotoxins in malt (Chapter II).
2. Survey the microbiota of international and Australian malt and barley from a wide range of environments and area by molecular techniques (Chapters III & V).
3. Predict what the desirable and undesirable microbial organisms and mycotoxins are and which likely to be present (Chapters II & IV).
4. Test the practical effect of these components on the brewing process and beer quality such as premature yeast flocculation (Chapter IV).

1.5 Experimental approach

Culture – independent molecular techniques, offering alternative, but not mutually exclusive ways for microbial identification and monitoring microbial communities population diversity, were used in this study. These methods are entirely molecular and, as such they provide a potential link between ecological processes and the organisms involved. In contrast, the application of traditional "wet plate" culture techniques in most studies (Ackerman 1998, Noots *et al* 1999, Petters *et al* 1988) are potentially biased towards the selective enrichment of fast growing microorganisms adapted to high substrate concentrations that can potentially represent a minor fraction of the resident microbial community. The incorporation of molecular information into diversity studies, therefore offers the possibilities of defining microbial communities accurately, studying microbial – host interaction directly in the environment and identifying ecologically active groups (Amann *et al* 1995, Pang and Mitchell 2005).

1.5.1 rRNA gene techniques to monitor microbial diversity in barley and malt

1.5.1.1 rRNA genes as biomarkers

The use of genetic markers to investigate microbial communities has provided a new approach to studies in microbial ecology. Of the genetic markers employed for such studies, genes that provide information regarding the presence or absence of a phenotypic trait and those indicating phylogenetic relationships among organisms have been extensively used (Liesack *et al* 1997). The small subunit ribosomal gene i.e., 16S rRNA gene in bacteria is most extensively used because: it contains both conserved and variable regions (Figure 1.9), contains enough sequence information to be used as a phylogenetic marker, is a dominant cellular constituent and lacks horizontal transfer (Liesack *et al* 1997, Muyzer and Ramsing 1995, Seguritan and Rohwer 2001, Woese 1987).

The D1/D2 region in yeast and filamentous fungi (Figure 1.10) refers to the variable domain of the large subunit ribosomal gene or the complete small subunit and is approximately 600 bases in size. The D1/D2 domains at the 5′ end of the large subunit (26 or 28S) rRNA gene show a high degree of inter specific sequence variation for yeasts and other fungi and are therefore frequently used for identification as well as in phylogenetic studies (Fell *et al* 2000, Kurtzman and Robnett 1998, Sugita and Nishikawa 2003). The sequencing databases of the D1/D2 sequences are now available for almost all currently recognised ascomycetous and basidiomycetous yeasts (Guffogg *et al* 2004, Kurtzman and Robnett 1995, 1997, 1998). This extensive available database makes the task of species identification much easier (Kurtzman 2001, Starmer *et al* 2001, Wesselink *et al* 2002).

Figure 1.9: Schematic of bacterial 16S rDNA showing conserved and hyper variable regions. Bact27F (5' AGA GTT TGA TCM TGG CTC AG 3') corresponds to positions 9–27 of the *Escherichia coli* 16S rDNA. Bact1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3') corresponds to positions 1492–1514 of the *Escherichia coli* 16S rDNA. The approximate sites for hyper variable regions (V1-V3) are shown as boxes.

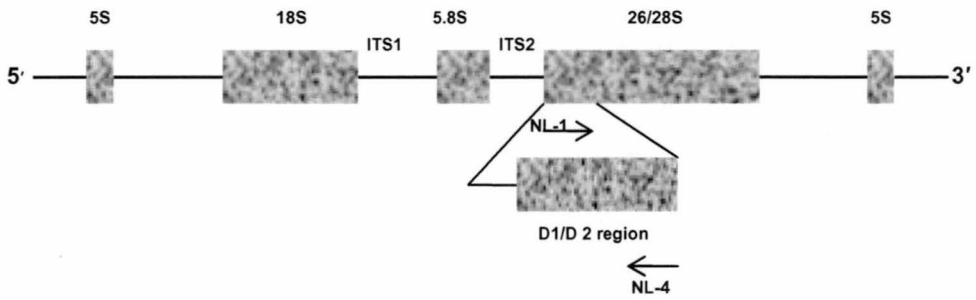


Figure 1.10: Schematic of fungal rRNA operon and location of D1/D2 region.

1.5.1.2 Clone libraries

Perhaps the most sensitive approach to assessing microbial diversity is assembling rRNA gene clone libraries of microbial assemblages. After DNA has been extracted from environmental samples clone libraries may be constructed in three ways (shot gun cloning, cloning of rRNA after reverse-transcriptase-PCR and direct cloning of PCR amplified rDNA), the most popular approach is the cloning and sequencing of rDNA amplification products. After PCR amplification of community rDNA, fragments are cloned into commercially available sequence ready vectors (Theron and Cloete 2000). Clones may then be sequenced. Sequences can then be compared to one another or others using sequence databases (e.g. genbank) so as to assess microbial community diversity. In complex communities it is necessary to sequence very large numbers of clones in order to gain insight into community diversity (Kemp and Aller 2004). Clone libraries, thus offer the highest resolution in assessing natural microbial community diversity, but are very time consuming, expensive, and also can be affected by unintended selection biases.

1.5.1.3 Terminal restriction fragment length polymorphism (TRFLP) – fingerprinting technique

Terminal restriction fragment length polymorphism (TRFLP) analysis is a method for rapid profiling of mixed populations of a homologous amplicon. It combines restriction fragment analysis of a PCR-amplified gene marker with automated sequencing gel technology (Figure 1.11). One or both PCR primers are labelled at the 5′ end with fluorescent dyes, in order that the terminal restriction fragments of the digested amplicon can be detected and quantified (Clement *et al* 1998, Liu *et al* 1997, Marsh 1999).

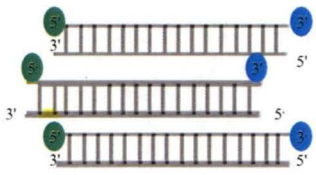
Since difference in the sizes of TRFs reflect differences in the sequences of rRNA genes (i.e. sequence polymorphisms), phylogenetically distinct populations of organisms can be resolved. Thus, the pattern of TRFs is a composite of DNA fragments with unique lengths that reflects the composition of the numerically dominant populations in the community. Being a high throughput fingerprinting technique TRFLP analysis has been applied extensively to the analysis of fungal

ribosomal genes (Genney *et al* 2006, Johnson *et al* 2004, Kennedy *et al* 2005, Liliya *et al* 2005) and bacterial 16S rRNA gene (Hullar *et al* 2006, Katsivela *et al* 2005, Noll *et al* 2005, Pérez-Piqueres *et al* 2006, Thies *et al* 2007). No doubt there are other possible procedures like denaturing gradient gel electrophoresis (DGGE), which have been used successfully to explore microbial diversity in barley malt ecosystem (Laitila 2007). However, TRFLP was considered to offer the greatest amount of information with a relatively large number of samples.

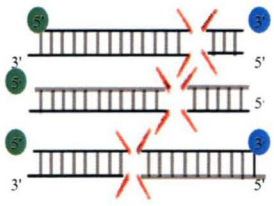
Barley/malt sample.

Genomic DNA extraction using FastDNA Spin Kit for soil.

PCR with fluorescently labelled primers
(27F & 1492R for bacteria and NL1 &NL4 for fungi).



Restriction digestion with endonucleases (*Hae*III, *Msp*I & *Rsa*I for bacteria and *Hae*III, *Hinf*I & *Rsa*I for fungi).



Electrophoresis of fluorescently labelled terminal fragments on Beckman Coulter CEQ8000 automated sequencer.

Data processing using T-Align and statistical analysis of TRFs using Primer v6 softwares.

Figure 1.11: Outline of the TRFLP method.

While TRFLP shares problems inherent to any PCR-based method like formation of heteroduplexes, chimeras, deletion mutants, point mutants, PCR bias to 1:1 ratio in multitemplate PCR (Kanagawa 2003, Lueders and Friedrich 2003, Qiu *et al* 2001, Terahara *et al* 2004, von Wintzingerode *et al* 1997) it has been shown to provide a facile means to assess changes in microbial community structure on temporal and spatial scales by monitoring the gain or loss of specific fragments from the profiles (Franklin and Mills 2003, Lukow *et al* 2000, Mummey and Stahl 2003). When coupled with rRNA gene clone library construction and clone sequencing, additional specific information on the composition of microbial communities can be obtained (Lindahl *et al* 2007, Singh *et al* 2007, Widmer *et al* 2006). Like any molecular approach TRFLP has both its advantages and disadvantages (Blackwood 2006, Marsh 1999, Muyzer 1999):

Advantages of TRFLP

- Automated, sensitive and rapid, can process multiple samples in a short time span.
- The use of intra-lane markers with a different fluorescent dye, which facilitates sample-to-sample comparison.
- Highly reproducible.
- Digital data available, making statistical analysis and comparison possible.
- Not limited to universal markers such as rRNA.
- High resolution can identify 60 – 80 unique terminal fragments.

Disadvantages of TRFLP

- No phylogenetic information can be obtained, have to do clone libraries or compliment with other molecular microbiological techniques.
- TRFs are not phylogenetic species thus direct comparison with databases may not yield useful information.
- Many species can share the same terminal length of the fragment even when optimal restriction enzymes are used.
- For unknown sequences selection of restriction enzymes can be difficult.
- The equipments used are expensive.

1.5.2 Immunoaffinity purification – Technique for analysis of mycotoxins

Because of the potential hazards that mycotoxins pose it is vital that the method used for their analysis should be precise and reliable. One such widely used method is purification by immunoaffinity columns before chromatographic analysis (Gilbert and Anklam 2002, Scott and Trucksess 1997). The immunoaffinity columns are used effectively to clean-up complex matrices and allow isolation and concentration of a specific toxin. The protocol involves addition of a sample extract to the column containing the immunoaffinity matrix; comprising a solid phases (e.g. agarose bead) to which anti-mycotoxin antibodies are covalently-coupled. The toxin in the sample binds to the corresponding immobilized antibodies. Subsequent steps involve removal of the unbound matrix components, including any coextractants, elution of the toxin by changing the solvent composition and finally, detection of the toxin using analytical techniques (Patel 2004). Alternatively, the mycotoxin bound to the column can be eluted and measured directly by fluorometry, based on the intrinsic fluorescence of mycotoxins or, quantified by using HPLC (Stroka *et al* 2000) and MS (Rosenberg *et al* 1998).

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Chapter II - Improving the cost efficiency of quality assurance screening for mycotoxins in malting barley

Chapter III – Microbial diversity of barley malt grown under different environmental conditions, in diverse geographic locations

3.1 Introduction

Barley malt is to beer as grapes are to wine (Goldammer 2008). The most extensive use for barley malt worldwide is as a source of fermentable sugars for alcoholic fermentations. Some 10% of the world barley crop is used, after malting, for the production of beer. Malt forms the perfect base for making wort, the liquid extracted from malt that is fermented into beer. Malt gives varying flavour, colour and body to beer depending on the type of malt being used (Bamforth and Barclay 1993).

Barley for malt is grown in a diverse range of environments and geographic locations. These include sub-arctic Scandinavia to near the equator; in the mountains of Ethiopia and in South America; from below sea level near the Dead Sea to great altitudes in the Andes and Himalayas; from humid, temperate regions, like western Europe to dryland areas in parts of North America, to irrigated areas in deserts, such as the Sahara (Hunter 1962, Briggs 1978, Rasmusson 1985). Production areas occur outside the humid tropics such as in Australia and the grain is traded widely. In Australia barley is grown as a "winter" crop in arid, temperate and intermediate climate regions with mostly winter rainfall resulting in dry maturation and harvest conditions. These conditions result in dry barley (<13% moisture) for storage and for subsequent malting. Such conditions maintain the germinative vigour of the barley and inhibit the growth of microbes during storage. Such advantages in part contribute to Australia's supply of around 32% of the world malting barley trade, ranking number one in world malting barley export (http://www.barleyaustralia.com.au). In this export trade, Australia has a reputation for bright and clean barley, suggestive of a low microbial load.

Grains are normally colonised by a wide variety of microbes; bacteria, yeasts and filamentous fungi (Flannigan 2003). These mixed populations are difficult to control and elimination is not possible or perhaps desirable (Laitila 2008) in a practical sense. These microbes have both positive and negative effects on grain quality in the field, in storage and at various stages during the malting process and on the quality of the resulting beer (Etchevers *et al* 1977, Evans *et al* 1999, Haikara and Home 1991, Laitila 2008, Laitila *et al* 2002, Laitila *et al* 2007, McMullen *et al* 1997, Sarlin *et al* 2005, Schehl *et al* 2007, Schwarz *et al* 1996, Schwarz *et al* 2001, van Nierop *et al* 2004).

Microbiota associated with grains vary in response to barley growing location (Birgitte *et al* 1996, Follstad and Christensen 1962), climatic conditions (Backhouse and Burgess 2002, Doohan *et al* 2003, Krstanović *et al* 2005,), malting techniques (Flannigan *et al* 1982) and storage and handling conditions (Hill and Lacey 1983, Laitila *et al* 2003). In addition, the detection and enumeration techniques used for analysis are important as to the microbes identified (Jarvis and Williams 1987, Rabie *et al* 1997). According to Flannigan (2003), barley provides as ecological niche for a diverse range of microorganisms, but the microbiota of different barleys are remarkably similar to each other, and to other cereals, in that the microbiota present is comprised by the same limited number of species as mentioned earlier in Chapter I, section 1.2 (refer to Flannigan 2003 and Noots *et al* 1999 for extra detail). Studies on fungi associated with South African barley malt reported that predominant species in South African barley malt were the same as those found elsewhere in the world, but the total numbers of fungi were significantly lower than those reported in the Northern Hemisphere (Rabie and Lübben 1993). This is not surprising, as the South African barley growing environment is in many ways similar to that of Australia, in that the grain maturation and harvest conditions are generally dry.

There is little information on the microbiota of Australian barley malt. Substantial amounts of data have been generated in America, Europe and in South Africa but the relevance of these studies to Australian malt may be limited. There is also presumably a significant body of data from ongoing monitoring and quality

assurance by the malting and brewing industries that is not in the public domain. So far, no study comparing the microbial community structures (of both bacteria and fungi) of Australian commercial malts, and limited studies of malts produced from barley grown under different geographical locations have been reported.

The majority of microbial studies associated with either barley or malt have been done using conventional "wet-plate" cultivation dependent methods, comparing quantitative changes in microbial populations. These conventional microbial cultivation dependent methods are biased towards the selective enrichment of fast growing microorganisms adapted to high substrate concentrations, which can potentially represent a minor fraction of the resident microbial community. On the other hand, cultivation independent PCR – based fingerprinting techniques have been developed for different ecosystems which enable the study of microbial diversity, structural composition and dynamics in greater depth. The use of one such technique, PCR- DGGE has been demonstrated to be a useful tool to monitor population dynamics in the malting ecosystem by Laitila *et al* (2007). In this study PCR-DGGE profiling revealed three different and previously undescribed uncultured Gram-positive bacteria, as well as Gram-negative *Agrobacterium* species that form part of the predominant bacterial communities in Finnish malts.

The objective of the present study was to compare the bacterial and fungal community structures of Australian commercial barley malts with international counterparts using terminal restriction fragment length polymorphism (TRFLP) fingerprinting supported by cloning and sequencing techniques. TRFLP analysis is a method for rapid profiling of mixed populations of a homologous amplicon. It combines restriction fragment analysis of a PCR-amplified gene marker with automated sequencing capillary electrophoresis technology. One or both PCR primers are labelled at the 5′ end with fluorescent dyes, in order that the terminal restriction fragments of the digested amplicon can be detected and quantified (Clement *et al* 1998, Liu *et al* 1997, Marsh 1999). Since differences in the sizes of TRFs reflect differences in the sequences of rRNA genes (i.e. sequence polymorphisms), phylogenetically distinct populations of organisms can be resolved. Thus, the pattern of TRFs is a composite of the DNA fragments with unique lengths that reflects the composition of the numerically dominant

populations in the community. Being a high throughput fingerprinting technique, TRFLP analysis has been applied extensively to the analysis of fungal ribosomal genes (Genney *et al* 2006, Johnson *et al* 2004, Kennedy *et al* 2005, Liliya *et al* 2005) and bacterial 16S rRNA genes (Hullar *et al* 2006, Katsivela *et al* 2005, Noll *et al* 2005, Pérez-Piqueres *et al* 2006, Thies *et al* 2007). While TRFLP shares problems inherent to any PCR-based method (Lueders and Friedrich 2003, Qiu *et al* 2001, Terahara *et al* 2004, von Wintzingerode *et al* 1997), it has been shown to provide a facile means to observe changes in microbial community structure on temporal and spatial scales by monitoring the gain or loss of specific fragments from the profiles (Franklin and Mills 2003, Lukow *et al* 2000, Mummey and Stahl 2003). When coupled with rRNA gene clone library construction and clone sequencing, additional specific information on the composition of microbial communities can be obtained (Lindahl *et al* 2007, Singh *et al* 2007, Widmer *et al* 2006).

3.2 Materials and Methods

3.2.1 Sample collection and preparation

A total of 34 Australian commercial malt samples were collected from different malt houses representing malt produced from barley grown in the different cropping zones and of different commercial varieties (Baudin, Gairdner, Grimmett, Schooner and Sloop) during the year 2005. International malt samples source country; regional association and sample number are shown in Table 3.1. Collected samples were stored in airtight bags at room temperature before grinding. Samples were ground in a Cyclone Sample Mill using a 0.1 mm screen (UDY Corporation, CO, USA) and stored immediately at -20°C until used for DNA extraction. Cross contamination between samples was avoided by blowing high pressure dry air through the grinding mill and collection container in between the samples and taking only the middle portion of the ground sample from the container for analysis.

Table 3.1: Detail of malt samples used in this study.

Geographic region	Countries included	No. of samples
Australasia (Australia)*	Australia	34
Eastern Europe (E. Europe)	Hungary, Russia, Slovakia, Ukraine	10
North America (N. America)	Canada, United States of America	3
Northern Europe (N. Europe)	Denmark, Finland, Ireland, Sweden, United Kingdom	9
Southern Africa (S. Africa)	South Africa	7
South America (S. America)	Argentina, Brazil, Chile	5
Western Europe (W. Europe)	Austria, Belgium, France, Germany, Switzerland	8

* Abbreviated and used there on.

3.2.2 DNA extraction and PCR

Genomic DNA from ground samples (0.1g) was extracted in duplicate with the FastDNA® Spin Kit for Soil (Q-Biogene, CA, USA) according to the manufacturer's instructions except that the samples were homogenised with a Restsch MM300 bead beater (Retsch GmbH, Haan, Germany) at 30/s frequency for 4 minutes. Immediately after extraction DNA samples were stored at -20°C until further use.

Extracted DNA were PCR amplified using bacterial 16S rRNA 5′ D3 WellRED dye-labelled 27F (AGA GTT TGA TCM TGG CTC AG) forward and 5′ D4 WellRED dye-labelled 1492R (TAC GGY TAC CTT GTT ACG ACT T) reverse primer pair (Sigma-Proligo, TX, USA) as described by Gurtler and Stanisich (1996). Each 60 µl reaction mixture contained 30 µl of 2 × ImmoMix Red™, 22.5 µl of ultra pure 18.2 MΩ DNAase/RNAase – free water (Bioline, NSW, Australia), 3 µl of each forward and reverse primers (10 pica mole) and 1.5 µl genomic DNA template. Thermocycling consisted of an initial denaturation at 95°C for 10 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min. The final extension was at 72°C for 10 min.

5′ D3 WellRED dye-labelled NL1 forward (GCA TAT CAA TAA GCG GAG GAA AAG) and 5′ D4 WellRED dye-labelled NL4 (GGT CCG TGT TTC AAG ACG G) reverse primers (Esteban *et al* 2006, O'Donnell 1993) specific to the D1/D2 domain of fungal 26/28S rRNA gene were used to study the impact of

geographical origin on malt fungal communities. The PCR mixture and thermocycling conditions were the same as described above except that the number of thermocycles was increased to 35. To check the purity and size of PCR amplicons, 5 µl of each reaction mixture was run on 1.5% agarose gel (w/v) stained with 500 ng/ml ethidium bromide. The PCR product was then purified using UltraClean™ PCR Clean-up Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and visualised again on a 1.5% agarose gel to detect any loss during purification and purification efficiency.

3.2.3 TRFLP analysis of bacterial and fungal communities

Microbial community fingerprint patterns (based on 16S rRNA and 26/28S rRNA genes for bacterial and fungal communities respectively) were generated by TRFLP analysis. Aliquots of purified DNA were digested with *Hae*III, *Msp*I and *Rsa*I (for bacterial PCR samples) and *Hae*III, *Hinf*I and *Rsa*I (for fungal PCR samples) (NewEngland Biolabs® Inc., Ipswich, MA, USA) restriction enzymes at 37°C for 3 h on a thermocycler and the reactions were stopped by a further incubation at 80°C or 65°C depending upon the restriction enzyme used for 20 min. Three restriction enzymes were used to eliminate false positives and pseudo-terminal restriction fragments (Egert and Friedrich 2003) which can occur when using only one restriction enzyme. The digested labelled fragments were then cleaned by ethanol precipitation using 3M sodium acetate (pH 5.2) with glycogen as a carrier molecule. Cleaned fragments were eluted in 30 µl formamide sample loading solution and 0.25 µl of Beckman Coulter size standard 600 (Beckman Coulter Australia Pty Ltd., NSW, Australia). Fragments were obtained by electrophoresis on Beckman Coulter CEQ8000 automated sequencer using modified Frag-4 method that involved an injection of 2.0 kV for 30 s, and was run at a capillary temperature of 50°C at 4.8kV for 90 min. Terminal restriction fragments (TRFs) obtained were analysed using the Beckman Coulter fragment analysis package version 8.0. Profiles were generated for each sample replicates based on relative area (abundance) of peaks whereby a peak height threshold was set to 5% and only TRFs present with a size between 60 and 640 bp regions were used for further analysis.

3.2.4 Statistical analysis of TRF data

Raw fragment data for bacterial and fungal communities obtained from Beckman Coulter CEQ8000 genetic analysis system were imported into Microsoft Excel. As samples were processed in duplicates right from the beginning hence to obtain a single fragment data set a free to use web based software called T-Align (http://inismor.ucd.ie/~talign/index.html) was used where fragments were binned with a 1.0 base confidence that also culled any fragments not present in duplicate samples (Smith *et al* 2005). The resultant data set were then imported into the multivariate statistical software package, Primer v6 (Primer-E Ltd, Plymouth Marine laboratory, UK) and a similarity matrix of relative abundance data was calculated utilising the Bray-Curtis coefficient (Bray and Curtis 1957). Bray – Curtis coefficient is a coefficient used to determine sample similarities based on organism abundances. It is widely employed in multivariate analysis of community assemblage data. It reflects differences between two samples due to both differing community composition and/or differing total abundance. A one-way analysis of similarity (ANOSIM) was used to examine the statistical significance of any relationship present between sample groups. ANOSIM tests the null hypothesis that the average rank similarity between samples within a group is the same as the average rank similarity between samples between groups i.e. there is no difference in microbial community composition of malt samples of different geographic origins. ANOSIM is based on the rank similarities between samples and produces a test statistic (R) which can range from -1 to 1. ANOSIM R value of 1 indicates that samples from a location are more similar to each other than to any samples from another location, whereas an R value of 0 indicates that there is more variation within a group than between the two groups being compared and thus the null hypothesis is true. A level of significance (p value) is also produced for the analysis using permutation analysis (n=999) (Clarke 1993, Clarke and Warwick 2001, Rees *et al* 2004).

Non-metric multidimensional scaling plots (MDS) were used to build a visual representation of the relative similarities/dissimilarities between the sample groups (on the basis of geographic location). MDS ordination is an iterative algorithm that involves a goodness of fit estimate, in this case the stress value of the final plot. A stress value greater than 0.2 indicates that the plot is close to

random, a stress less than 0.2 indicates a useful 2 dimensional plot, and less than 0.1 indicates the plot shows relationship less influenced by artefactual data (Clarke 1993, Clarke and Warwick 2001, Rees *et al* 2004).

Similarity percentage (SIMPER) analysis was used to identify TRFs that characterised each group. This analysis calculates the average contribution of individual TRFs to the average similarity within a group (90% in this study). Terminational restriction fragments contributing 90 percent group similarities were identified by virtually digesting clone library sequences (section 3.2.5) with restriction enzymes used in this study using BioEdit software version 7.0.5.3 (Hall 1999) (Ibis Biosciences, Carlsbad, CA, USA). This statistical routine also computes the average dissimilarity between all pairs of inter-groups and then breaks this average down into separate contributions from each peak to the average dissimilarity (90% in this study) (Clarke 1993, Clarke and Warwick 2001, Rees *et al* 2004).

Multivariate dispersion (MVDISP) indices were also calculated to examine within group (geographic location) heterogeneity. Samples sets with large dispersion index values possess high sample-to-sample variability in comparison to other sample sets (Clarke 1993, Clarke and Warwick 2001, Rees *et al* 2004).

For all the above statistical analyses (separately for fungi and bacteria) the relative abundance data of forward and reverse TRFs obtained from all the three restriction enzymes were pooled together.

3.2.5 Clone library construction and sequencing

Three fungal clone libraries were generated from DNA extracted from two different Australian malt samples, and one was constructed from DNA extracted from one of the N. American malt samples. One bacterial clone library for one of the Australian malt samples was also made in this study.

Bacterial 16S rRNA gene and fungal D1/D2 domain of 26/28S rRNA gene amplicons obtained from selected samples were cloned in *Escherichia coli* using TOPO TA Cloning® Kit (Invitrogen, Carlsbad, USA), following the

manufacturer's instructions. The correct insert size in each clone was checked by vector targeted PCR (primer M13F and M13 R) and agarose gel electrophoresis. PCR amplicons were purified using UltraClean™ PCR Clean-up Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). About 48 fungal clones per sample and 96 bacterial clones were selected for sequencing. Selected clones were sent to and sequenced for this work by Macrogen, Korea with the BigDye Terminator Ready Reaction mix sequencing reaction kit using the vector specific T7 or T3 promoters as primers (both T7 and T3 primers were used to get a complete sequence of the bacterial PCR product); with sequence reactions ran on an automated DNA sequencer (Applied BioSystems). Raw sequence files (*.ab1) were imported into BioEdit where chromatograms were analysed and sequence fragments were aligned against reference sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/Blast) (Altschul *et al* 1990).

3.3 Results

3.3.1 Analysis of fungal communities

TRFLP analysis and cloning and sequencing of D1/D2 domain of 26/28S rRNA gene was used to compare fungal community structure of malts obtained from different malt houses produced from barley grown in diverse geographical locations. Figure 3.1 shows the typical electropherograms for the fungal and bacterial primers from one representative malt sample.

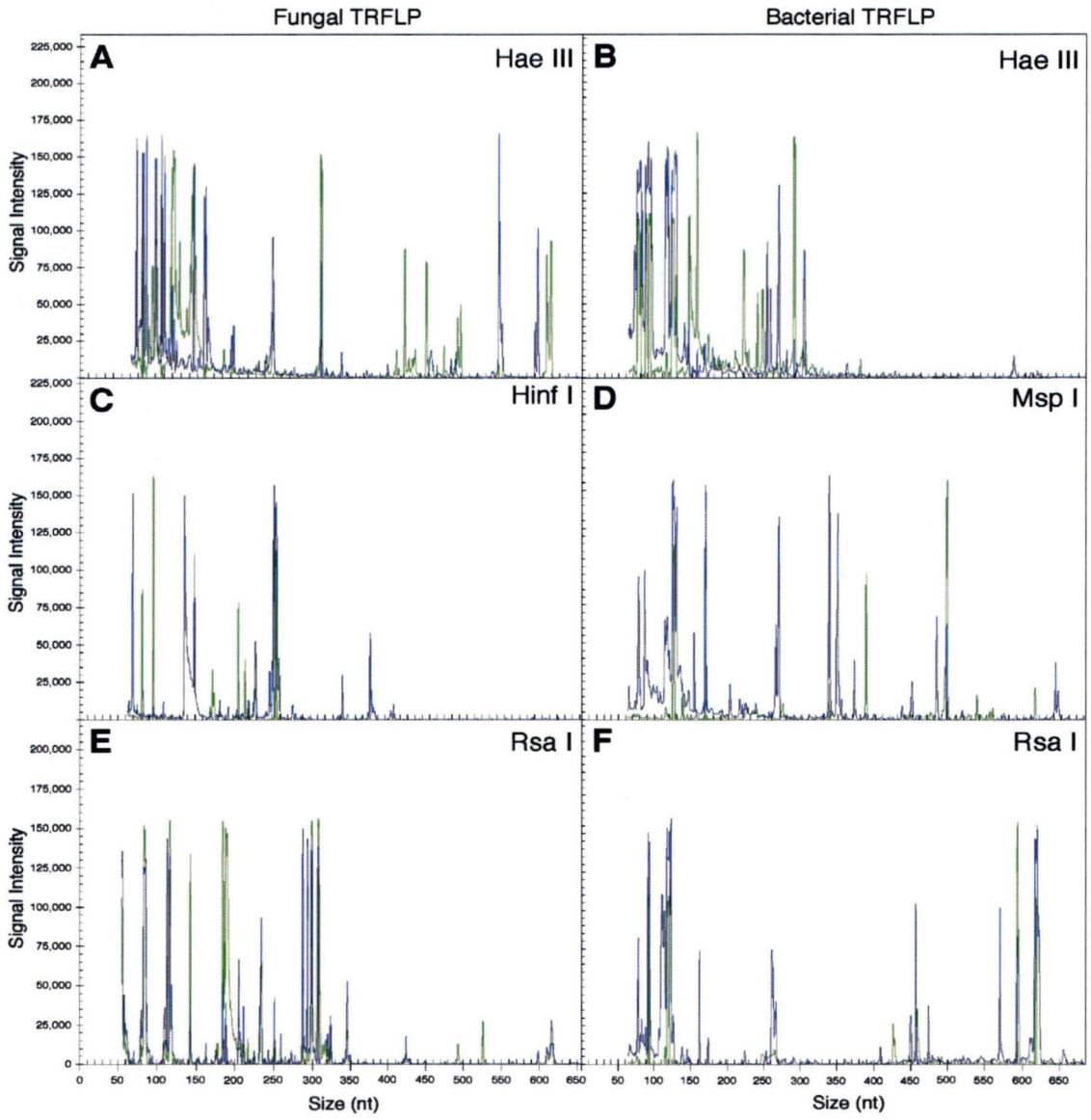

Figure 3.1: Typical TRFLP profiles from a single malt sample of the D1/D2 domain of the fungal 26/28S rRNA gene (A, C & E) and the bacterial 16S rRNA gene (B, D & F) partial sequences digested with *Hae*III, *Hinf*I, *Msp*I and *Rsa*I restriction enzymes. Green peaks represent forward fragments and blue peaks represent reverse fragments.

3.3.1.1 Similarity analysis of fungal TRFs

In order to test for the impact of malt sample origin on variation in the similarity data, ANOSIM was applied. Pairwise comparison of geographically different malt groups (Table 3.2) revealed that the R values were >0.4 in ten (Australia – N. America, N. Europe – E. Europe, N. Europe – N. America, E. Europe – S. America, W. Europe – E. Europe, W. Europe – S. America, N. America – E. Europe, N. America – W. Europe, N. America – S. America and S. Africa – E. Europe) out of a total 21 group pairs with a significance level of ≤0.03, indicating that malt produced in these geographical locations resulted in significant differences in fungal community structures. N. American malts were significantly different from all other groups except S. Africa whereas, S. African malts were statistically different only from E. Europeans in terms of fungal community profile (Table 3.2).

Table 3.2: ANOSIM values (one - way analysis) comparing the similarity between pairs of different geographical malt groups (fungi).

Pair of geographic groups	Global R statistic	*p* value
Australia (34) * – E. Europe (10)	0.168	0.04
Australia (34) – N. America (3)	**0.497**	**0.01**
Australia (34) – N. Europe (9)	0.234	0.01
Australia (34)– S. Africa (7)	0.231	0.05
Australia (34)– S. America (5)	-0.170	0.89
Australia (34) – W. Europe (8)	0.030	0.37
E. Europe – S. America	**0.649**	**0.002**
N. America – E. Europe	**0.656**	**0.007**
N. America – S. Africa	0.230	0.15
N. America – S. America	**0.579**	**0.02**
N. America – W. Europe	**0.866**	**0.006**
N. Europe – E. Europe	**0.480**	**0.001**
N. Europe – N. America	**0.449**	**0.03**
N. Europe – S. Africa	0.142	0.06
N. Europe – S. America	0.076	0.23
N. Europe – W. Europe	0.030	0.27
S. Africa – E. Europe	**0.573**	**0.001**
S. Africa – S. America	0.189	0.05
S. Africa – W. Europe	0.399	0.003
W. Europe – E. Europe	**0.874**	**0.001**
W. Europe – S. America	**0.679**	**0.002**

*Figures in parentheses show number of malt samples analysed in each group.

3.3.1.2 Description of community pattern of fungal TRFs

Overall, the combined MDS plot (Figure 3.2 A) for all geographical malt groups showed large dispersion and overlapping of groups. A closer look at pairwise MDS plots (Figure 3.2 B – 3.2 K) for groups with significant ANOSIM R values revealed group-wise distinct clustering of Australian (Figure 3.2 B), N. European (Figure 3.2 C and D), E. European (Figure 3.2 D, F, H, I and K), W. European (Figure 3.2 E, I and J), S. American (Figure 3.2 G, J and K) and S. African (Figure 3.2 H) malts. Whereas, N. American malts show less tendency to group together and thus possessed more heterogenous communities (Figure 3.2 B-C and E-G). Small stress values i.e. <0.1 for all the MDS plots (except for Figure 3.2 A and B) indicate a good ordination with no real prospect of misleading interpretation. The stress values for Figure 3.2 A and B were slightly larger (ranging between 0.16 – 0.18), but still provided a meaningful picture of fungal community patterns.

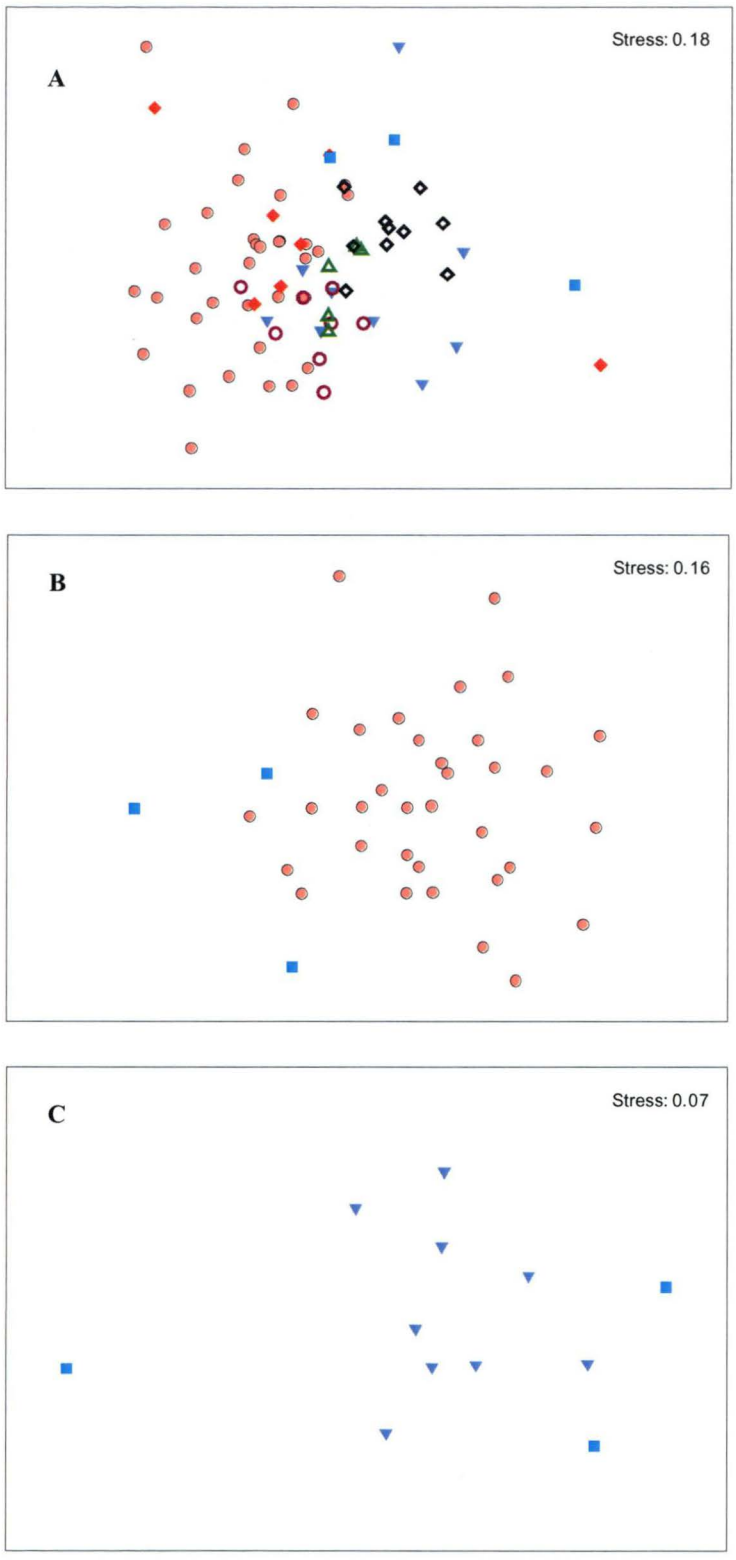

Figure 3.2: MDS plots showing the relative similarities between the Australian (●) & N. European (▼), N. American (■), S. African (◆), W. European (○), E. European (◇) & S. American (△) malt samples. All samples are shown in figure A, for clarity the Australian (●) & N. American (■) in (B) and N. American (■) & N. European (▼) in (C) samples are also presented separately.

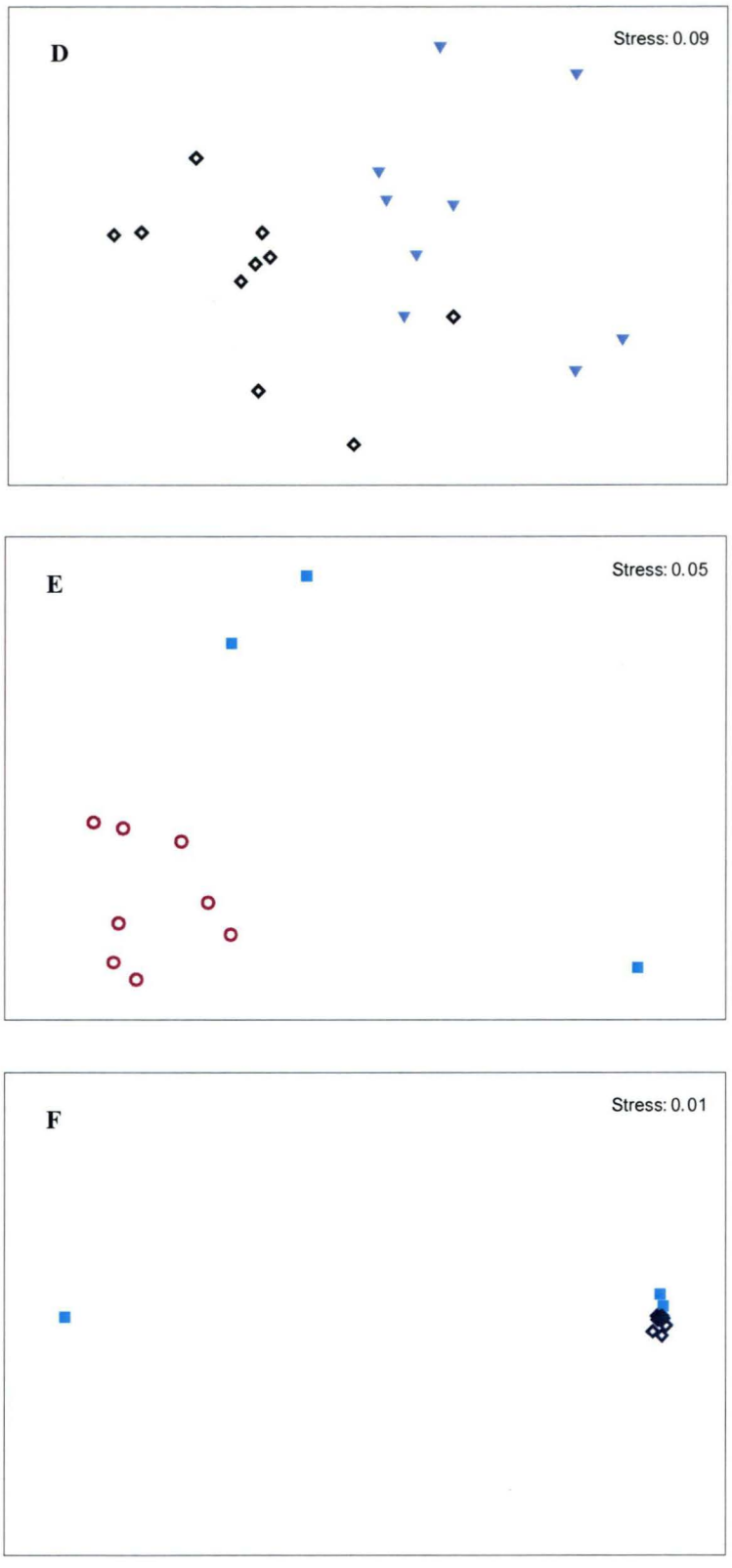

Figure 3.2 contd.: MDS plots showing the relative similarities between the N. European (▼) & E. European (◇) (D), N. American (■) & W. European (○) (E) and N. American (■) & E. European malt samples (◇) (F).

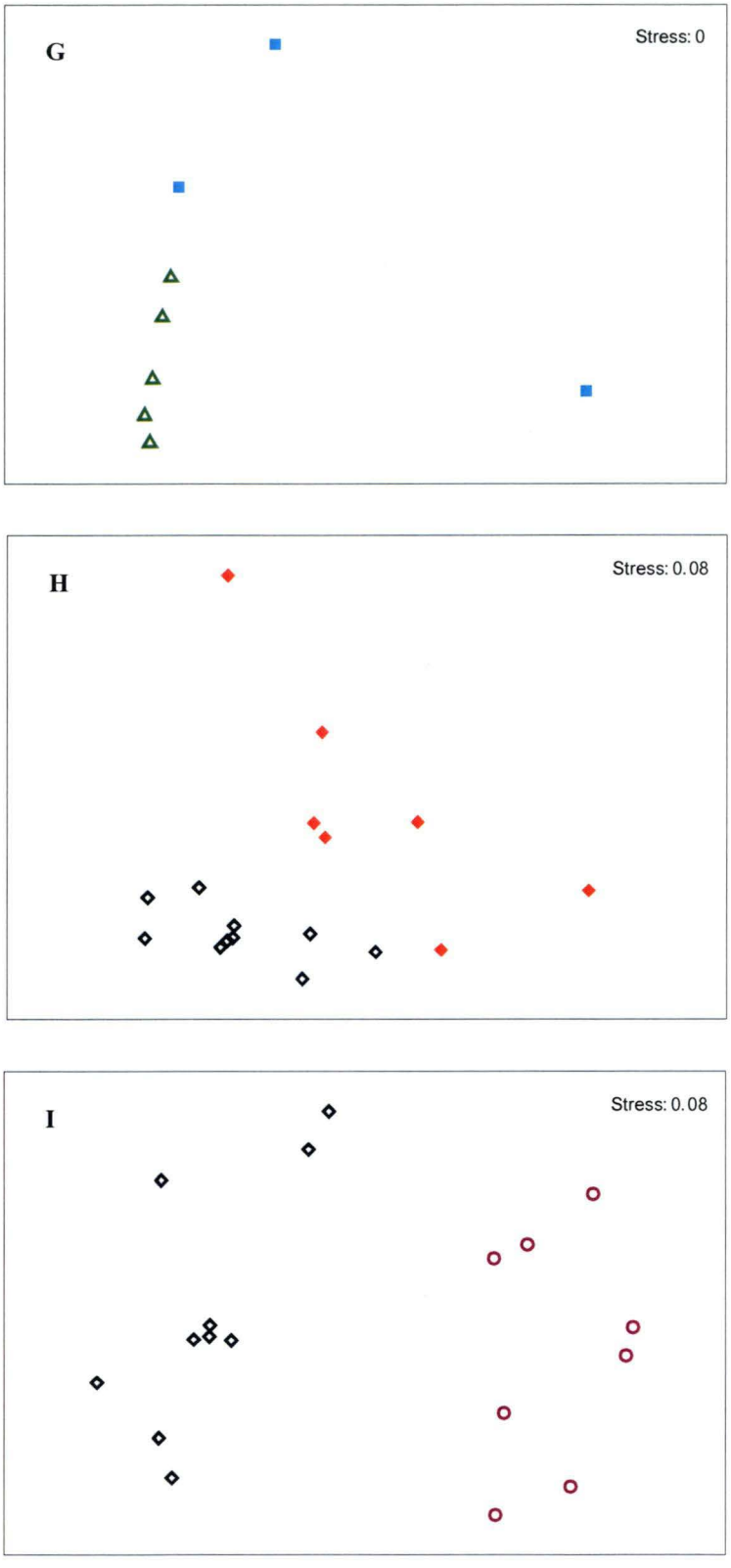

Figure 3.2 contd.: MDS plots showing the relative similarities between the N. American (■) & S. American (△) (G), S. African (◆) & E. European (◇) (H) and W. European (○) & E. European malt samples (◇) (I).

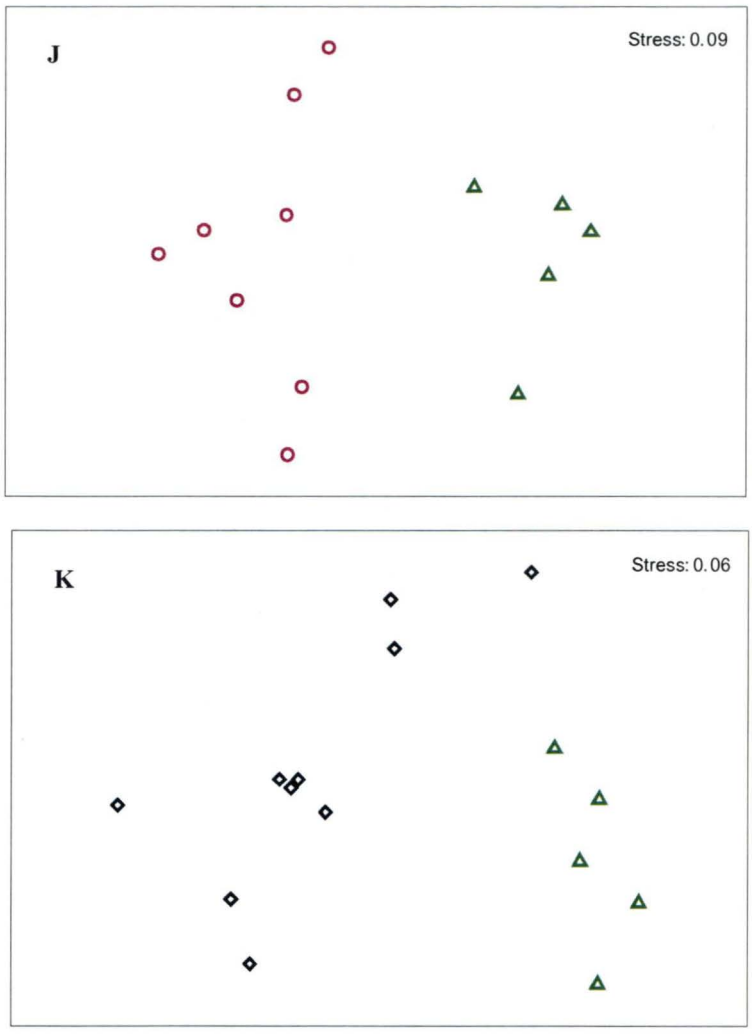

Figure 3.2 contd.: MDS plots showing the relative similarities between the S. American (△) & W. European (○) (J) and S. American (△) & E. European (◇) (K).

3.3.1.3 Similarity percentages and dispersion indices of fungal TRFs

With SIMPER analysis the average percent similarity and the number of TRFs needed to explain 90% of this similarity within a group was analysed (Figure 3.3 and Table 3.3). Average similarity within a group was the lowest in N. American malts (23.8%) and was the highest in S. American malts (53.9%). Considering the large number of samples in the Australian malts group, the average similarity within this group was comparable (35.9%) with other groups that had fewer sample numbers. The number of TRFs constituting this similarity was the highest in Australian malts (Figure 3.3 and Table 3.3). Relative dominance by fewer

fungal groups resulted in lower number of TRFs in N. American malts (Figure 3.3 and Table 3.3).

Table 3.3: SIMPER analysis of the fungal terminal restriction fragments contributing 90% of similarity/dissimilarity within/between group/groups.

Geographical group	Average similarity within group (%)
Australia (34)*	35.9 (82)**
E. Europe (10)	53.2 (57)
N. America (3)	23.8 (22)
N. Europe (9)	38.9 (57)
S. Africa (7)	31.5 (64)
S. America (5)	53.9 (45)
W. Europe (8)	48.8 (52)
Pair of geographical groups	**Average dissimilarity between groups (%)**
Australia – E. Europe	65.7 (176)
Australia – N. America	72.4 (164)
Australia – N. Europe	67.3 (190)
Australia – S. Africa	68.5 (216)
Australia – S. America	61.3 (162)
Australia – W. Europe	63.9 (185)
E. Europe – S. America	57.4 (109)
N. America – E. Europe	63.6 (115)
N. America – S. Africa	73.6 (163)
N. America – S. America	65.2 (102)
N. America – W. Europe	71.7 (134)
N. Europe – E. Europe	63.1 (142)
N. Europe – N. America	70.7 (133)
N. Europe – S. Africa	66.7 (190)
N. Europe – S. America	59.1 (130)
N. Europe – W. Europe	57.3 (159)
S. Africa – E. Europe	67.7 (176)
S. Africa – S. America	65.8 (160)
S. Africa – W. Europe	65.7 (191)
W. Europe – E. Europe	63.8 (139)
W. Europe – S. America	56.5 (128)

*Figures in parentheses show number of malt samples analysed in each group.

**Figures in parentheses represent the number of observed TRFs.

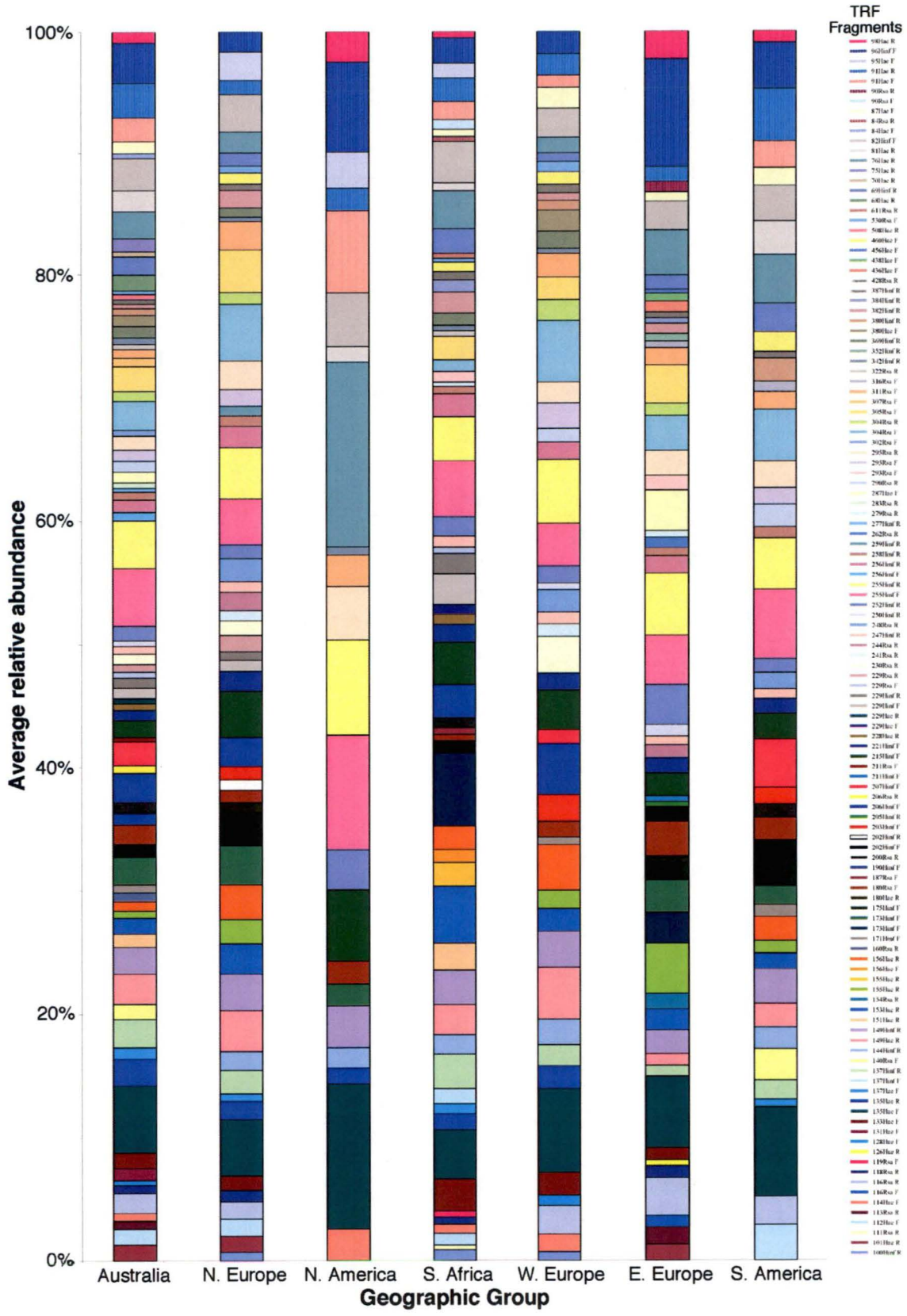

Figure 3.3: Average relative abundance of the observed fungal TRFs in different geographical malt groups.

Lower homogeneity and higher within group variability was also indicated by higher dispersion indices for the N. European (0.946), Australian (1.094), S. African (1.367) and N. American (1.664) malts as compared to S. American (0.141), E. European (0.288) and W. European (0.298) malts.

When different geographical malt groups were compared among themselves the N. American malts showed the greatest dissimilarity from S. African malts (73.6%), closely followed by the Australian (72.4%), W. European (71.7%) and N. European (70.7%) malts (Table 3.3).

3.3.1.4 Assignment of sequences to fungal TRFLP fragments

The sequences obtained from clone libraries (prepared in this study and studies for Chapters IV and V) were virtually digested in BioEdit software using the same three restriction enzymes used to cleave the PCR products from malt DNA. The lengths of these theoretical TRFs were calculated and sequences were assigned to peaks found in the electropherograms, considering a ± 1bp in the sizes of TRFs (Table 3.4). Almost all the clones could be assigned to one or the other peaks except for 26/28S rRNA gene partial sequences of *Tiarosporella tritici* and *Filobasidium globisporum* found in clone library 1 (Figure 3.4 A) and 2 (Figure 3.4 B), respectively. In all, the known clones could be assigned to 40 – 45% of the TRFLP peaks.

Table 3.4: Identification of fungal TRF peaks present in the electropherograms using clone library data presented in Figure 3.4 and Table 3.5.

TRF	Filamentous fungi/yeast identified
96*Hinf* F, 118*Rsa* R, 187*Rsa* F	*Alternaria alternata*
96*Hinf* F, 101*Hae* R, 118*Rsa* R, 187*Rsa* F	*Alternaria malorum*
175*Hinf* F	*Aureobasidium pullulans*
137*Hae* F, 387*Hinf* R	*Candida anglica*
248*Rsa* R	*Candida boidinii**
180*Rsa* F, 215*Hinf* F	*Candida intermedia*
380*Hae* F	*Candida silvae**
384*Hinf* R	*Candida solani*
206*Rsa* R	*Cryptococcus macerans*
68*Hae* R, 69*Hinf* R, 91*Hae* F, 95*Hae* F	*Cryptococcus magnus*
69*Hinf* R, 91*Hae* F	*Cryptococcus oeirensis*
229*Hinf* R, 279*Rsa* R, 316*Rsa* F	*Cryptococcus* sp.
75*Hae* R	*Cryptococcus victoriae*
135*Hae* F, 155*Hae* R, 380*Hinf* R 382*Hinf* R	*Davidiella tassiana*
100*Hinf* R, 187*Rsa* F	*Drechslera erythrospila**, *Pyrenophora tritici-repentis**, *Sporobolomyces roseus*
87*Hae* F, 116*Rsa* F, 119*Rsa* F, 151*Hae* R, 153*Hae* R, 160*Rsa* R, 211*Hinf* F, 295*Rsa* F, 342*Hinf* R	*Geotrichum* sp.
118*Rsa* R, 387*Hinf* R	*Glonium pusillum*
173*Hinf* F, 305*Rsa* F, 307*Rsa* F	*Issatchenkia siamensis*
91*Hae* R, 295*Rsa* R, 302*Rsa* F, 384*Hinf* R	*Issatchenkia* sp.
96*Hinf* F, 118*Rsa* R, 187*Rsa* F	*Phoma medicaginis*
304*Rsa* R, 387*Hinf* R, 456*Hae* F	*Pichia anomala*
76*Hae* R, 90*Rsa* F	*Saccharomyces exiguus**

* Identified from clone libraries of barley and/or malt samples studied in Chapters IV and V.

3.3.1.5 Sequence analysis of fungal clones

26/28S rRNA gene clone libraries were constructed for the fungal community from malts. For each of the three clone libraries, 48 clones per library were selected randomly for sequencing of 600-640bps. In all, 105 (46 for library 1, 39 for library 2 and 20 for library 3) good quality sequences were obtained and further analysed. Comparison using the National Centre for Biotechnology Information database (BLAST algorithm) most of the sequences were confirmed as 26/28S rRNA fungal genes. These studied sequences showed 99 – 100% homology to known 26/28S rRNA fungal sequences in the database (Table 3.5). All sequences were grouped into *Ascomycota* and *Basidiomycota* phyla. The distribution of different genera of fungi in each clone library is presented in Figure 3.4. Overall, the malt samples used for clone libraries were dominated by *Cryptococcus*; *Alternaria*, *Aureobasidium, Davidiella*, *Geotrichum*, and

Issatchenkia were common in both the Australian malt clone libraries, but *Phoma*, *Pichia*, *Sporobolomyces* and *Tiarosporella* genera were present in clone library 1 and *Candida* and *Filobasidium* were present in the second clone library only (Figure 3.4 A and B). Relatively few genera (*Alternaria*, *Cryptococcus*, *Davidiella* and *Glonium*) constituted the N. American malt sample clone library (Figure 3.4 C).

Table 3.5: Sequence match of D1/D2 domain of fungal 26/28S rRNA gene sequences isolated from malt samples.

Filamentous fungi/yeast	GenBank Accession No.	Similarity	Source isolated	Citation
Ascomycota				
Alternaria alternata strain: IFM 53969	AB363761.1	100%	Barley malt associated	Petters *et al* (1988)
Alternaria malorum strain STE-U 4571	AY251081.2	99%	Soil, grains, fruits and grass litter associated	Braun *et al* (2003)
Alternaria malorum var. *polymorpha* strain STE-U 4570	AY251080.2	99%	Soil, grains, fruits and grass litter associated	Braun *et al* (2003)
Aureobasidium pullulans	AB104687.1	100%	Barley malt associated	Petters *et al* (1988)
Aureobasidium pullulans strain UWFP 993	AY213693.1	99%	Culture collection	Rakeman *et al* (2005)
Aureobasidium pullulans strain VTT D-1013	DQ377656.1	100%	Barley malt associated	Laitila *et al* (2006)
Candida anglica strain VTT C-04517	DQ377632.1	100%	Barley malt associated	Laitila *et al* (2006)
Candida intermedia strain VTT C-04520	DQ377635.1	99%	Barley malt associated	Laitila *et al* (2006)
Candida solani strain VTT C-04528	DQ377642.1	100%	Barley malt associated	Laitila *et al* (2006)
Davidiella tassiana strain CBS 572.78	DQ289799.2	100%	Plant associated	Crous *et al* (2006)
Davidiella tassiana strain STE-U 5101	AY251078.2	100%	Plant associated	Braun *et al* (2003)
Geotrichum sp. DTQ-26.3	DQ640273.1	99%	Associated with natural fat and oils degradation	Quyen *et al* (unpublished)*
Geotrichum sp. DTQ-LP20.11	EF025925.1	99%	Associated with natural fat and oils degradation	Quyen *et al* (unpublished)*
Glonium pusillum CBS:119348	EU552134.1	99%	Plant associated	Marincowitz *et al* (2008)
Issatchenkia sp. XM03C	EU293430.1	99%	Marine yeast	Zhang (unpublished)*
Issatchenkia siamensis strain: EF6	AB439220.1	100%	Mangrove forests yeast	Limtong (unpublished)*
Phoma medicaginis strain CBS 533.66	EU167575.1	99%	Plant associated	Simon (unpublished)*
Pichia anomala isolate 33	EU285512.1	100%	Marine yeast	Song *et al* (unpublished)*
Tiarosporella tritici strain CBS 118719	DQ377941.1	99%	Plant associated	Crous *et al* (2006)

*Please refer to GenBank with quoted accession no. for authors' detail.

Table 3.5 contd.: Sequence match of D1/D2 domain of fungal 26/28S rRNA gene sequences isolated from malt samples.

Filamentous fungi/yeast	GenBank Accession No.	Similarity	Source isolated	Citation
Basidiomycota				
Cryptococcus sp. VTT C-04545	DQ377666.1	99%	Barley malt associated	Laitila *et al* (2006)
Cryptococcus macerans strain VTT C-04538	DQ377662.1	100%	Barley malt associated	Laitila *et al* (2006)
Cryptococcus magnus HG1-1	AY242120.1	100%	Plant associated	Yang and Wang (2003)
Cryptococcus oeirensis strain HB 1220	AM160646.1	99%	Insect associated	Molnar & Prillinger (unpublished)*
Cryptococcus victoriae isolate ESAB12	AJ749830.1	100%	Food associated	Calhelha *et al* (unpublished)*
Filobasidium globisporum strain VTT C-04511	DQ377680.1	99%	Barley malt associated	Laitila *et al* (2006)
Sporobolomyces roseus isolate ESAB18	AJ749836.1	99%	Food associated	Calhelha *et al* (unpublished)*
Sporobolomyces roseus strain HB 1216	AM160644.1	100%	Insect associated	Molnar & Prillinger (unpublished)*

*Please refer to GenBank with quoted accession no. for authors' detail.

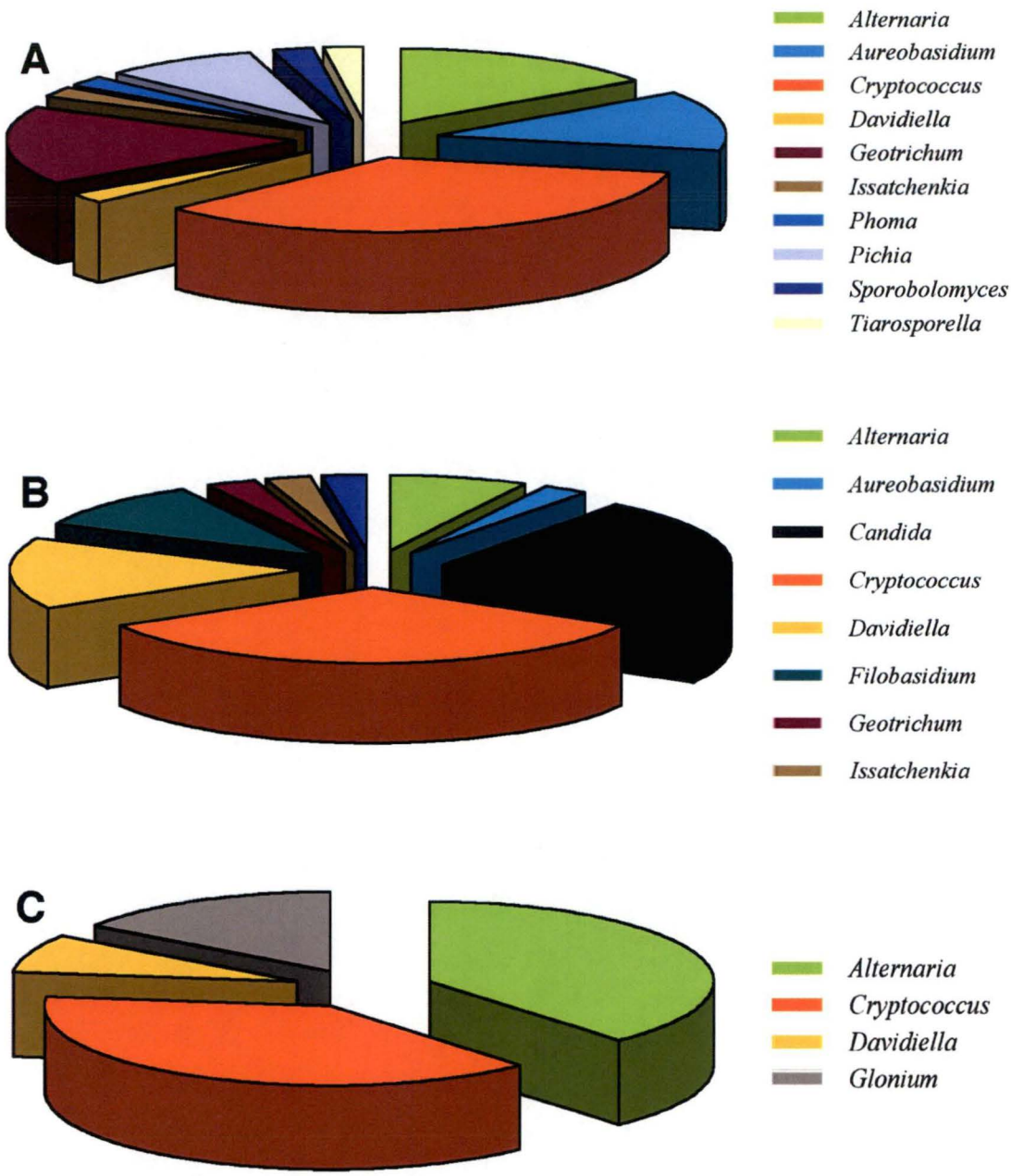

Figure 3.4: Relative percentages of fungal genera identified from the sequencing of D1/D2 domain of the 26/28S rRNA gene sequences isolated from the Australian (A and B) and N. American malt samples (C).

3.3.2 Analysis of bacterial communities

The effect of malt origin on bacterial community structure was explored using TRFLP and cloning and sequencing analysis of partial 16S rRNA gene sequences.

3.3.2.1 Similarity analysis of bacterial TRFs

One-way ANOSIM results for each pair of geographical malt group are presented in Table 3.6. Paired comparisons of the Australian malts bacterial community demonstrated that there were no significant differences between the Australian malts and its international counterparts. Significant differences were observed when N. American group was compared with E. European and W. European malt groups (R statistic was>0.7 and p value was ≤ 0.006).

Table 3.6: ANOSIM values (one - way analysis) comparing the similarity between pairs of different geographical malt groups (bacteria).

Pair of geographic groups	Global R statistic	p value
Australia (34) * – E. Europe (10)	-0.143	0.93
Australia (34) – N. America (3)	0.187	0.15
Australia (34) – N. Europe (9)	0.018	0.43
Australia (34)– S. Africa (7)	0.067	0.28
Australia (34)– S. America (5)	0.018	0.43
Australia (34) – W. Europe (8)	-0.181	0.94
E. Europe – S. America	0.386	0.01
N. America – E. Europe	**0.751**	**0.003**
N. America – S. Africa	-0.012	0.44
N. America – S. America	0.292	0.14
N. America – W. Europe	**0.774**	**0.006**
N. Europe – E. Europe	0.221	0.007
N. Europe – N. America	0.254	0.11
N. Europe – S. Africa	0.082	0.17
N. Europe – S. America	0.278	0.03
N. Europe – W. Europe	0.259	0.005
S. Africa – E. Europe	0.150	0.05
S. Africa – S. America	-0.025	0.53
S. Africa – W. Europe	0.241	0.001
W. Europe – E. Europe	0.214	0.008
W. Europe – S. America	0.254	0.04

*Figures in parentheses show number of malt samples analysed in each group.

3.3.2.2 Description of community pattern of bacterial TRFs

When the TRFLP results for N. American and E. European malts were plotted on MDS, location wise grouping was observed. Malt samples from E. Europe grouped together quite distinctly compared to the N. American samples (Figure 3.5 A). A similar trend was observed when W. European and N. American malt samples were plotted on MDS (Figure 3.5 B) indicating differences in bacterial community structures between them.

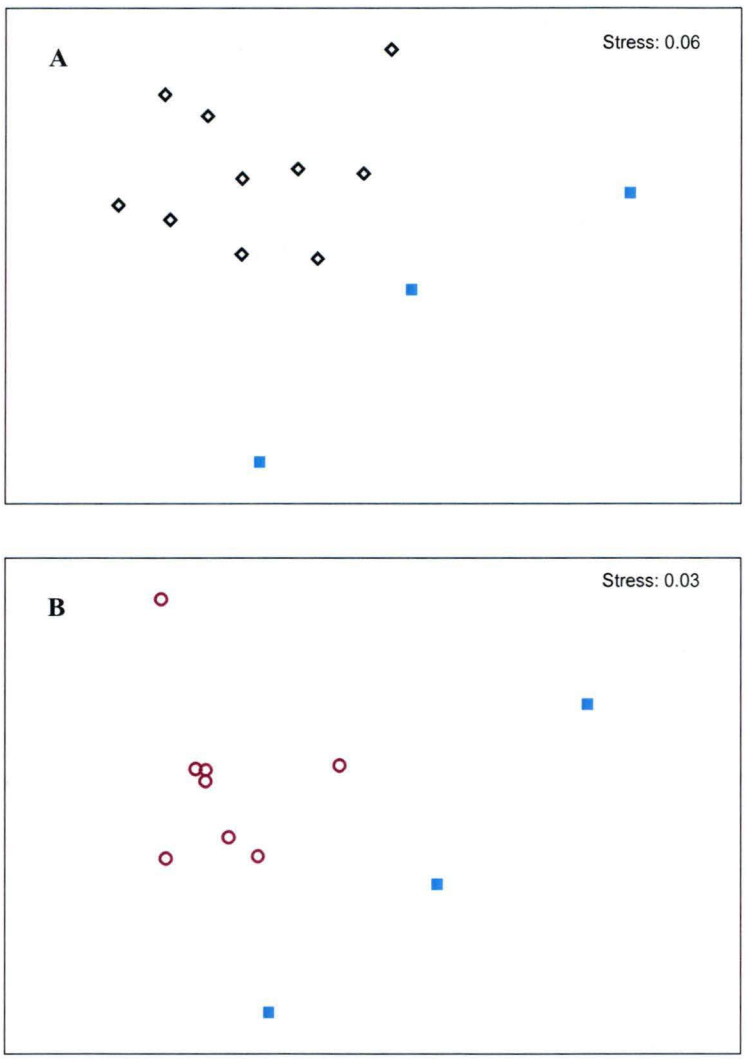

Figure 3.5: MDS plots showing the relative similarities between the bacteria of N. American (■) & E. European (◇) (A) and N. American (■) & W. European (○) (B) malt samples.

3.3.2.3 Similarity percentages and dispersion indices of bacterial TRFs

Around 60% within group similarity was observed in E. European and W. European malt samples and 48% in Australian, N. American and S. American malt groups (Table 3.7). As in the fungal analysis, the highest number of bacterial TRFs contributing 90% within group similarity was observed in the Australian malts and the lowest in N. American malt samples (Figure 3.6 and Table 3.7).

Higher within group similarity in E. European and W. European malts was also explained by lower dispersion indices (0.369 and 0.306 respectively) observed for these groups as compared to N. Europe (0.745), Australia (1.091), S. America (1.107), N. America (1.13) and S. Africa (1.216).

Pair-wise average dissimilarities for malt associated bacterial communities (Table 3.7) were lower than those observed for fungal communities. The highest value was observed for the N. American and S. American (54.7%) malt groups, closely followed by N. American and Australian (54.4%) malt groups.

Table 3.7: SIMPER analysis of the bacterial terminal restriction fragments contributing 90% of similarity/dissimilarity within/between group/groups.

Geographical group	Average similarity within group (%)
Australia (34)*	48.6 (75)**
E. Europe (10)	60.4 (60)
N. America (3)	48.5 (40)
N. Europe (9)	55.2 (53)
S. Africa (7)	44.3 (61)
S. America (5)	48.7 (57)
W. Europe (8)	62.2 (63)
Pair of geographical groups	**Average dissimilarity between groups (%)**
Australia – E. Europe	48.2 (144)
Australia – N. America	54.4 (133)
Australia – N. Europe	51.3 (137)
Australia – S. Africa	53.6 (154)
Australia – S. America	51.6 (146)
Australia – W. Europe	47.8 (144)
E. Europe – S. America	46.0 (125)
N. America – E. Europe	49.7 (108)
N. America – S. Africa	54.0 (122)
N. America – S. America	54.7 (112)
N. America – W. Europe	51.0 (111)
N. Europe – E. Europe	44.8 (115)
N. Europe – N. America	48.9 (106)
N. Europe – S. Africa	50.2 (126)
N. Europe – S. America	51.1 (116)
N. Europe – W. Europe	45.8 (115)
S. Africa – E. Europe	47.4 (138)
S. Africa – S. America	53.4 (137)
S. Africa – W. Europe	49.6 (136)
W. Europe – E. Europe	41.4 (121)
W. Europe – S. America	44.6 (122)

*Figures in parentheses show number of malt samples analysed in each group.

**Figures in parentheses represent the number of observed TRFs.

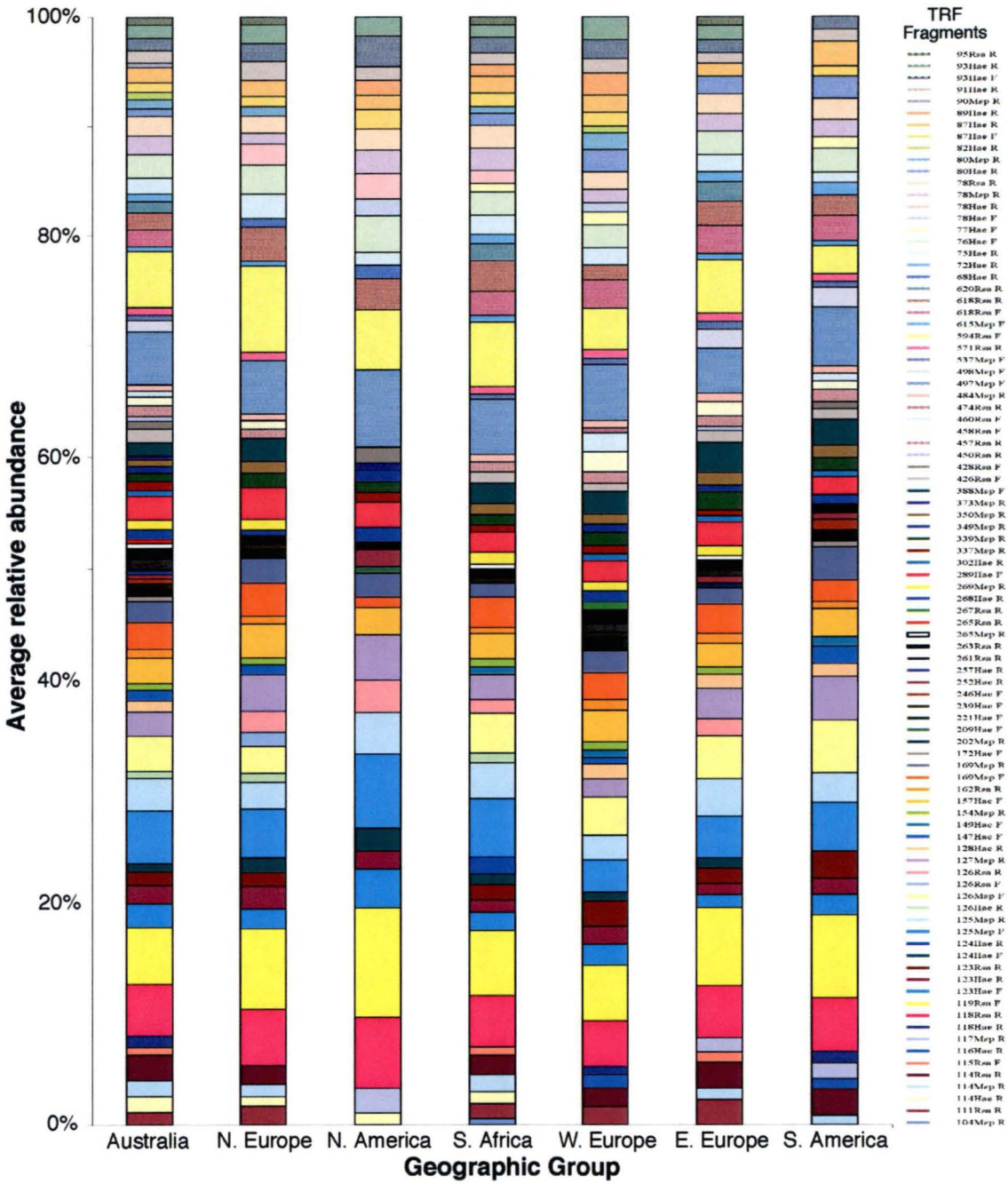

Figure 3.6: Average relative abundance of the observed bacterial TRFs in different geographical malt groups.

3.3.2.4 Assignment of sequences to bacterial TRFLP fragments

Similar to fungal sequences, theoretical fragments obtained from virtual digestions of clone library sequences were assigned to TRF peaks found in the electropherograms. The known clones could be assigned to 28 – 35% of the TRFLP peaks. Almost all except *Kineococcus radiotolerans, Pseudomonas* sp. OK-5, *Pseudomonas oleovorans* and *Sphingomonas aerolata* sequences had theoretical TRFLP patterns that were found in one or more electropherograms (Table 3.8). *In silico* cleavage of uncultured bacterial clones resulted in TRFLP patterns different from those found in the TRFLP analysis of malt samples analysed in this study.

Table 3.8: Identification of TRF peaks present in the electropherograms using bacterial clone library data presented in Figure 3.7 and Table 3.9.

TRF	Bacteria identified
89*Hae* R, 91*Hae* R	*Acinetobacter calcoaceticus, Leuconostoc citreum*
78*Hae* R, 124*Hae* R	*Arthrobacter ardleyensis**
75*Hae* R, 80*Msp* R	*Arthrobacter globiformis*
75*Hae* R, 78*Hae* R, 80*Msp* R, 124*Hae* R	*Arthrobacter protophormiae*
75*Hae* R, 78*Hae* R, 80*Msp* R , 123*Rsa* R, 124*Hae* R, 125*Msp* F, 126*Rsa* R	*Arthrobacter* sp., *Arthrobacter nicotianae*
68*Hae* R, 118*Rsa* R	*Brachybacterium rhamnosum*
72*Hae* R, 78*Msp* R, 123*Hae* R	*Curtobacterium flaccumfaciens*
72*Hae* R, 78*sp* R	*Curtobacterium* sp. C01
426*Rsa* F, 428*Rsa* F, 497*Msp* F	*Enterobacter* endosymbiont of *Metaseiulus occidentalis, Enterobacter cloacae, Enterobacter hormaechei, Enterobacter sakazakii, Erwinia tasmaniensis, Escherichia senegalensis, Pantoea agglomerans, Pantoea ananatis**
75*Hae* R	*Frigoribacterium* sp.
126*Hae* R	*Lactococcus lactis, Enterobacter* endosymbiont of *Metaseiulus occidentalis, Enterobacter hormaechei, Enterobacter sakazakii, Erwinia tasmaniensis, Escherichia senegalensis, Pantoea agglomerans, Pseudomonas argentinensis, Pseudomonas fulva, Pseudomonas lutea, Pseudomonas putida, Pseudomonas rhizosphaerae*
78*Msp* R	*Leucobacter* sp.
119*Rsa* F, 257*Hae* R	*Massilia aurea*
72*Hae* R, 78*Msp* R, 458*Rsa* F	*Microbacterium hydrocarbonoxydans*
118*Hae* R	*Microbacterium* sp.
154*Msp* R	*Paenibacillus* sp.
118*Hae* R, 127*Msp* R	*Plantibacter agrosticola**
128*Hae* R	*Rhodococcus erythropolis**, *Kurthia gibsonii**

* Identified from clone library of malt samples studied in Chapter IV.

3.3.2.5 Sequence analysis of bacterial clones

16S rRNA gene clone library was constructed for one Australian malt sample. In total 96 clones were selected randomly for sequencing of about 1500 bases and 70 good quality sequences were further analysed. The NCBI BLAST results confirmed these sequences as partial sequences of 16S rRNA bacterial gene with 96 -99% similarity (Table 3.9). Further bacterial sequences were grouped into the phyla *Actinobacteria*, *Proteobacteria* and *Firmicutes*. The distribution of different bacterial genera in the clone library is presented in Figure 3.7. Nine uncultured bacterial clones were also found in this study, some of them had the similarity percentage less than 96% and thus were not included in the Table 3.9. Overall, *Proteobacteria* dominated the Australian malt sample, constituting nearly half (47.1%) of the clones, followed by *Actinobacteria* (32.8%) and *Firmicutes* (7.2%).

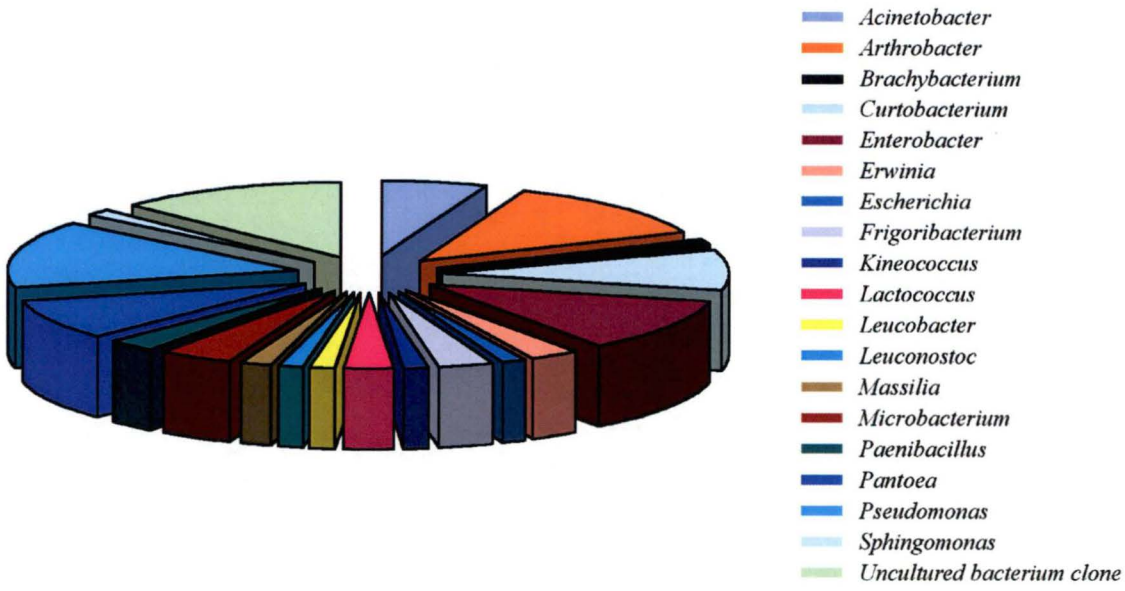

Figure 3.7: Relative percentages of bacterial genera identified from partial sequencing of 16S rRNA gene sequences isolated from an Australian malt sample.

Table 3.9: Sequence match of partial bacterial 16S rRNA gene sequences isolated from malt sample.

Bacteria	GenBank Accession No.	Similarity	Source isolated	Citation
Actinobacteria				
Arthrobacter sp. GOL01	AY940423.1	99%	Soil associated	Pan *et al* (unpublished)*
Arthrobacter globiformis	AB098573.1	97%	Compost associated	Hiraishi *et al* (2003)
Arthrobacter nicotianae strain SB42	AJ315492.1	98%	Cheese ripening Starter culture	Place *et al* (unpublished)*
Arthrobacter protophormiae strain m3	EU874451.1	97%	Malachite green degrading soil bacterium	Fang and Li (unpublished)*
Brachybacterium rhamnosum type strain LMG 19848T	AJ415376.1	98%	Phenolic compound degradation	Heyrman *et al* (unpublished)*
Curtobacterium sp. C01	EF411134.1	99%	Potato tube seedling associated	Shen *et al* (unpublished)*
Curtobacterium flaccumfaciens pv. *flaccumfaciens* type strain DSM 20129	AM410688.1	99%	Plant associated	Stackebrandt *et al* (unpublished)*
Frigoribacterium sp. R-25593	AM944034.1	99%	Marine bacterium	Vandecandelaere *et al* (unpublished)*
Kineococcus radiotolerans SRS30216	CP000750.2	98%	Host not specified	Copeland *et al* (unpublished)*
Leucobacter sp. 38	DQ406732.1	96%	Sewage bacterium	Li and Zong (unpublished)*
Microbacterium sp. SSL14	EU373326.1	98%	Plant associated	Cho *et al* (unpublished)*
Microbacterium hydrocarbonoxydans strain HNR08	EU373354.1	98%	Plant associated	Cho *et al* (unpublished)*
Uncultured actinobacterium clone NAB30	AY395034.1	98%	Phytophagous insect associated	Broderick *et al* (2004)
Firmicutes				
Lactococcus lactis subsp. *lactis* IL1403	AE006456.1	99%	Cheese starter culture	Bolotin *et al* (2001)
Lactococcus lactis strain HDDMM12	EU723831.1	98%	Hemp retting water	Cai *et al* (unpublished)*
Leuconostoc citreum KM20	DQ489736.1	96%	Kimchi (food) associated	Kim *et al* (2008)
Paenibacillus sp. HDDMM03	EU723825.1	98%	Hemp retting water	Cai *et al* (unpublished)*

*Please refer to GenBank with quoted accession no. for authors' detail.

Table 3.9 contd.: Sequence match of partial bacterial 16S rRNA gene sequences isolated from malt sample.

Bacteria	GenBank Accession No.	Similarity	Source isolated	Citation
Proteobacteria				
Acinetobacter calcoaceticus culture collection MTCC:9488	FM210755.1	99%	Host not specified	Banga and Tripathi (unpublished)*
Enterobacter cloacae subsp. *dissolvens* strain SREPS 3	EF025329.1	98%	Soil associated	Dave (unpublished)*
Enterobacter endosymbiont of *Metaseiulus occidentalis* clone pAJ239	AY753172.1	99%	Insect associated	Hoy and Jeyaprakash (2005)
Enterobacter hormaechei strain TMPSB-T10	EU047556.1	98%	Insoluble phosphate solublizer	Shrivastava and D'Souza (unpublished)*
Enterobacter sakazakii strain HDTL-01	EU693527.1	98%	Host not specified	Ge and Wang (unpublished)*
Erwinia tasmaniensis strain ET1/99	CU468135.1	98%	Plant associated	Kube *et al* (2008)
Escherichia senegalensis	AY217654.1	98%	Millet bran associated	Mbengue *et al* (unpublished)*
Massilia aurea type strain AP13T	AM231588.1	99%	Water associated	Gallego *et al* (2006)
Pantoea agglomerans strain BJ-Tobacco	AY849936.1	99%	Insect associated	Li (unpublished)*
Pantoea agglomerans strain PSB-UJ2-22	EU849108.1	98%	Plant associated	Huang (unpublished)*
Pantoea agglomerans	EU598802.1	97%	Plant associated	Ren *et al* (unpublished)*
Pseudomonas sp. OK-5	EF157292.1	99%	Soil associated	Cha *et al* (unpublished)*
Pseudomonas argentinensis strain HDDMG01	EU723817.1	99%	Hemp retting water	Cai *et al* (unpublished)*
Pseudomonas fulva strain Z58zhy	AM410620.1	98%	Sea water associated	Zhang and Li (unpublished)*
Pseudomonas lutea strain PSB2	EU184082.1	99%	Rhizospheric soil associated	Peix *et al* (unpublished)*
Pseudomonas oleovorans	AY623816.1	98%	Associated with herbicide degradation	Xu *et al* (2006)
Pseudomonas putida GB-1	CP000926.1	98%	Host not specified	Copeland *et al* (unpublished)*
Pseudomonas rhizosphaerae	AY152673.1	99%	Soil associated	Peix *et al* (2003)
Sphingomonas aerolata strain NW12	AJ429240.1	98%	Dust associated	Busse *et al* (2003)
Uncultured bacterium clone BANW664	DQ264610.1	97%	Ground water associated	DeSantis *et al* (2007)

*Please refer to GenBank with quoted accession no. for authors' detail.

3.4 Discussion

TRFLP analysis of rRNA gene fragments from natural microbial populations associated with malts was applied to investigate the geographical variations in the microbial community structures. This technique was demonstrated to be a useful tool to monitor spatial population dynamics in malts once optimised. TRFLP analysis was further complimented by cloning and sequencing to assist with the identification of dominant fragments within different TRFLP profiles. As reported by Laitila *et al* (2007) and Normander and Prosser (2000), barley DNA contamination was observed not only in bacterial but also in fungal DNA profiling. This problem was resolved by excluding barley chloroplast or mitochondrial rRNA gene derived peaks from TRFLP data before performing the statistical analyses.

Great microbial diversity in Australian malt samples was observed in this study which might be the result of the wide distribution of barley cropping regions in different agro climatic zones within Australia. Backhouse and Burgess (2002) and Backhouse *et al* (2004) have also reported spatial differences in the distribution of *Fusarium* spp. associated with cereals, not only between countries but between regions within a country such as Australia. In addition, the barley malt assessed in this study was sourced from different varieties, which might have contributed to the large within group heterogeneity. Different barley head architecture and potential pathogenesis resistance genes to certain microbial taxa could also account for these differences. Barley varietal differences have previously been found to significantly influence microbial populations and mycotoxin production (Perkowski *et al* 2003).

Statistical analysis of TRFLP data showed geographical differences in fungal community structure of Australian malts from those of N. American malts. These differences were the combined effect of both qualitative (type of fungi) as well as quantitative (relative abundance of different fungi) dissimilarities as observed from differences in number and relative abundance of TRFs in different samples. Geographical differences in fungal communities were also observed between other groups such as Europe and N. America, N. America and S. America as well

as within Europe. The South African malts were only different from the E. European counterparts. Significant differences in total numbers of fungi on S. African malts and Northern Hemisphere malts were also reported by Rabie and Lübben (1993). In addition, Ackermann (1998) observed lower fungal counts in S. African barley malts as compared to malts from other countries by using direct plating techniques. However, these authors emphasized that the predominant fungal species were the same on all types of malt regardless of their geographical origin.

In contrast, this extensive study found differences in type of fungi associated with different malts as indicated by differences in TRFs' size in different groups. Thus, suggesting the presence of both quantitative as well as qualitative differences in fungal populations associated with malts produced in different geographic regions. Presumably this contrast with previous studies is due to the application of the culture independent TRFLP analysis. These qualitative differences in addition to the quantitative ones are of great importance when the overall quality of malts is judged, as certain fungal species or strains are more deleterious in their effect on malt quality, such as production of mycotoxins (Burgess 1985, Esteban *et al* 2006, Frisvad *et al* 2005, Jestoi *et al* 2004, Kosiak *et al* 2003, Langseth *et al* 1997). Conversely some may be of a beneficial nature to malt quality such as *Geotrichum candidum* and *Rhizopus* sp. (Boivin and Malanda 1997, Dufait and Coppens 2002, Dziuba *et al* 2000), which can be used as malting starter cultures. This spatial displacement of harmful species or strains by competition from less harmful or beneficial species might contribute to the overall improved quality of some malt compared to others.

This investigation also noted the presence of a large number of yeasts (indicated in fungal clone libraries and corresponding TRFs) which might have contributed to the differences between malt groups. In previous studies, comparisons among barley and malt samples were mostly concentrated on culturable filamentous fungi and less attention has been paid to yeasts and yeast-like fungi. Exception being extensive work done by Laitila (2007) on barley malt associated yeasts characterization and further on their utilization in the malting process. Yeasts are the second most abundant microbes after bacteria in viable counts in pre-harvest

barley (Flanningan 2003), and are reported to survive during storage and malting (Clarke and Hill 1981, Haikara *et al* 1977, Flannigan *et al* 1982). Furthermore, malt associated yeasts have shown strong antagonistic activity and have been applied as natural biocontrol agents to restrict growth of harmful fungi (Dziuba and Foszczyńska 2001, Laitila *et al* 2006, Lefyedi and Taylor 2007).

The fungal cloning and sequencing data in this study revealed some similarity with fungal community previously mentioned in literature on microbiota of barley and malt. *Alternaria alternata* is a common airborne fungus which colonizes cereal crops such as barley in the field and is widely reported in Europe, N. America and S. Africa in literature (Ackermann 1998, Haikara *et al* 1977, Kosiak *et al* 2004 and Petters *et al* 1988). Likewise *Aureobasidium pullulans*, *Candida anglica*, *Candida intermedia*, *Candida solani*, *Geotrichum candidum*, *Issatchenkia orientalis, Phoma herbarum*, *Phoma sorghina, Pichia anomala*, *Cryptococcus* sp. VTT C-04545, *Cryptococcus macerans*, *Cryptococcus magnus*, *Cryptococcus victoriae*, *Filobasidium globisporum*, *Sporobolomyces roseus* (Douglas and Flannigan 1988, Flannigan 1969, Flannigan and Dickie 1972, Flannigan and Healy 1983, Flannigan *et al* 1982, Laitila *et al* 2006, Rabie *et al* 1997, Tuomi *et al* 1995) have been reported in the malting ecosystem.

In this study the application of the TRFLP and cloning approaches identified a number of novel fungal variations. These included some different strains of the previously reported species (*Aureobasidium pullulans* strain UWFP 993, *Sporobolomyces roseus* isolate ESAB18, *Sporobolomyces roseus* strain HB 1216) different species of the same already mentioned genera (*Alternaria malorum*, *Geotrichum* sp. DTQ-26.3, *Geotrichum* sp. DTQ-LP20.11, *Issatchenkia* sp. XM03C, *Issatchenkia siamensis*, *Cryptococcus oeirensis*) and even some new genera not previously identified to be associated with barley malt system (*Davidiella tassiana*, *Glonium pusillum*, *Tiarosporella tritici*). However, at 98% or lower similarity, going to the level of strain, may suggest that the clone is either a novel or unknown species. Most of the fungal genera, species or strains recorded in this study seemed to be widely associated with either anthropogenic plant-based systems, such as agriculture or natural ecosystems i.e. forests, except for

Issatchenkia sp. XM03C, *Pichia anomala* isolate 33 which are reported as marine yeasts (Table 3.5).

The cloning and sequencing approach was found to be a very useful technique in identifying the diversity within malt samples when correlated with TRFLP data. However, in this study only 40-45% of TRFs could be assigned to the corresponding fungi which suggested the need in future studies to construct further clone libraries, especially when large numbers of samples are studied as in this work, and greater numbers of clones per sample to fully capture the fungal diversity.

ANOSIM and MDS analysis of bacterial TRFLP data showed significant differences in bacterial community structures of N. American malts from E. European and W. European malts but no differences were observed between other locations. Bacterial diversity in terms of TRFs numbers seemed to be more consistent in groups than those observed in the fungal community suggesting less of an influence of geographical factors on bacterial community. This might be due to less host specificity and more cosmopolitan distribution of bacteria observed in nature as compared to fungi.

Cloning and sequencing results for bacterial identification revealed that proteobacteria were the dominant bacteria in malted barley. Similar results for Gram-negative bacteria (proteobacteria) dominance in indigenous microbial communities of malted barley were reported by Laitila *et al* (2007). Morris and Monier (2003) had also observed Gram-negative bacteria prevalence in plant based ecosystems. In contrast, Petters *et al* (1988) reported dominance of Gram-positive bacteria in screened malts, but they did mention *Arthrobacter* and *Pseudomonas* as predominant genera along with *Alcaligenes*, *Clavibacter*, *Erwinia* and *Lactobacillus* in overall barley malt production. Large proportions of *Arthrobacter* (12.8%) and *Pseudomonas* (14.3%) were also observed in the clone library in the present study.

Further, cloning and sequencing of bacterial 16S rRNA gene sequences revealed the presence of *Arthrobacter, Curtobacterium, Enterobacter, Erwinia,*

Escherichia, Leuconostoc, Microbacterium, Paenibacillus, Pantoea, and *Pseudomonas,* which have already been reported in the barley malt literature (Flannigan and Dickie 1972, Flannigan *et al* 1982, Haikara and Home 1991, Haikara *et al* 1977, Haikara *et al* 1993, Laitila *et al* 2007, Petter *et al* 1988). Although the aforementioned bacterial genera were also observed in this study, there were differences in the species members observed for most genera. In addition, some new genera; *Acinetobacter, Brachybacterium, Frigoribacterium, Kineococcus, Lactococcus, Leucobacter, Massilia, Sphingomonas,* were also observed in this study. As with fungi, most of the bacteria reported in this study are widely distributed in agroecosystems except for clones related to *Frigoribacterium* sp. R-25593 and *Pseudomonas fulva* strain Z58zhy, which are sea water associated (Table 3.9).

More bacterial diversity can still be expected as only one sample was used for cloning and sequencing and it has represented only 28 – 35% of the total TRFs observed in TRFLP profiles of different malt groups. These extra clone libraries to explain this diversity would form the basis of future studies.

3.5 Summary

An extensive investigation was undertaken by microbial DNA based fingerprinting of Australian malt made from barley grown in different regions. This was benchmarked against malting barley grown internationally by using terminal restriction fragment length polymorphism (TRFLP) analysis. This approach was supported by cloning and sequencing techniques to assess microbial population composition. The TRFLP approach was considered the most appropriate because it is comparatively rapid and cost efficient, and profiles from a large number samples can be assessed. The TRFLP approach used generic primers for the amplification of bacterial 16S rRNA gene and D1/D2 domain of the fungal 26/28S rRNA gene. Both qualitative and quantitative differences were observed in bacterial and fungal (especially) communities associated with malts produced in different geographical regions. The TRFLP and cloning and sequencing techniques revealed greater diversity in barley malt ecosystem than was anticipated from previous reports. Care should be taken while extrapolating

these results as this study was done with only limited numbers of malt samples from some geographic regions, especially N. America. In addition, only one cropping season was assessed with a small number of cultivars that might not be comparable.

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Chapter IV - Investigation of premature yeast flocculation using TRFLP and clone libraries

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Chapter V - Microbial community changes during malting - a study of Australian barley and malt

Chapter VI– General discussion and future directions

6.1 Introduction

This thesis investigated the microbial (bacteria, filamentous fungi, and yeast) composition of Australian barley and malts, and compared Australian malt with International malt. This detailed comparative microbial study of Australian barley and malt was conducted to fill a deficiency in the literature of the understanding microbial ecology of Australian barley and malt. Molecular ecological-relevant, culture independent techniques i.e. rRNA gene TRFLP and cloning and sequencing were successfully applied, as traditional culture – dependent approaches are known to underestimate the microbial species diversity. The improved understanding of barley/malt associated microbial structure gained from this work seeks to further enhance our knowledge of the implications of microbial associated malt quality characters such as premature yeast flocculation and occurrence of mycotoxins.

6.2 General discussion

Analysis of mycotoxins using immunoassays is a precise and reliable technique. Unfortunately, the cost of these IACs is relatively high, and manufacturers' do not generally recommend their reuse. Consequently, the relatively high cost involved with these assays reduces the inclination of maltsters or other grain users to undertake routine analysis of large number of samples, especially when the likelihood of these mycotoxins (i.e. DON and OTA) being present are low, such as with Australian barley and malts. A method was successfully developed and used to regenerate DON and OTA immunoaffinity columns for at least five reuses (Chapter II). Such recycling substantially reduces the cost of these routine assays for the malting and brewing industry. The column regeneration method developed for barley and malt, could also be used for mycotoxin analyses in other cereal based or products having similar matrices. The absence of DON and OTA in Australian barley and malts once again highlighted their relative microbial safety and quality.

The climate under which a barley crop is grown, combined with the malting protocols used to malt the barley grains, are known to influence the microbial community structure associated with these grains. Nevertheless no or little work has been done to simultaneously compare geographically diverse commercial malts especially in terms of bacteria, filamentous fungi and yeasts. According to Flannigan (2003), barley is an ecological niche for a diverse range of microorganisms, but the microbiota of different barleys are remarkably similar to each other, and to other cereals, in that the microbiota present is comprised of the same limited number of species (refer to Flannigan 2003 and Noots *et al* 1999 for detail). The studies carried out in Chapters III, IV, and V demonstrated that spatial distinctiveness has a great impact on microbial community structure associated with grains. Perhaps the previously observed similarity is an artefact of culture dependent methods initially used to assess diversity? It was shown that geographic distance between samples origin need not be large to result in differences in microbial community structure. Differences between countries or continents were also observed and regional differences within a country such as Australia, presumably due to divergent growing conditions, were sufficient to induce changes in community structure (Chapters III and V). Thus it would appear that climate under which the barley was grown is most likely a very important factor in determining the microbial community composition of barley grains.

It was found that both filamentous fungi and yeasts showed greater spatial variability than bacteria. Despite yeast being the second most abundant microbes colonising the barley grains, relatively little is being reported about them in literature as compared to filamentous fungi and bacteria (Flannigan 2003, Laitila *et al* 2006). TRFLP and cloning and sequencing techniques revealed greater diversity in barley and malt than was anticipated from previous reports including Flannigan 2003, Laitila 2007, Noots *et al* 1999, Petters *et al* 1988. Studies reported in Chapters III, IV, and V recorded a number of novel fungal as well as bacterial taxa. These included some different strains of the previously reported species, different species of the already mentioned genera, and also some new genera not previously identified to be associated with barley malt system such as

the fungal genera: *Davidiella*, *Glonium*, *Tiarosporella, Udeniomyces;* and the bacterial genera: *Acinetobacter, Brachybacterium, Frigoribacterium, Kineococcus, Lactococcus, Leucobacter, Massilia, Sphingomonas* genera. However, at 98% or lower microbial clone similarity, diversity variation begins to indicate differences between strains and suggest that the clone is either a novel or unknown species. Most of the bacterial and fungal genera, species or strains recorded in these studies are widely associated with either anthropogenic plant-based systems, such as agricultural or natural ecosystems i.e. forests. These conclusions underline the importance of new and sophisticated research techniques in studying the microbial diversity of complex, highly dynamic microbial ecosystems such as the growing and malting of barley.

Although rRNA gene-based molecular techniques are highly sensitive, precise and rapid, they are relatively expensive. Additionally, data analysis is relatively complicated and time-consuming, so that data acquisition errors could potentially lead to misinterpretation of the results. As previously mentioned in this thesis (section 4.2.1, Chapter IV), there were some limitations in the number of samples assessed for some sample groups, a lack of comparison between different crop seasons, barley varieties or more extensive clone library assessment (Chapters III, IV, and V), that were not undertaken due to time and monetary constraints to fully permit the exhaustive exploration of microbial diversity. Despite these practical limitations, this thesis has significantly advanced the understanding of microbial population diversity associated with malting enabling improved focus for future investigations.

Equally as the barley growing location affect the grain-associated microbial communities, so do the malting protocols used to malt barley in diverse malt houses (Armstrong and Bendiak 2007, Axcell *et al* 1986, Axcell *et al* 2000, Blechová *et al* 2005, Griggs *et al* 2008, Gyllang and Martinson 1976, Noots *et al* 1999, O' Sullivan *et al* 1999, Petters *et al* 1988, Sasaki *et al* 2008, van Nierop *et al* 2004, Yoshida *et al* 1979). Malting is an important process during which the microbial community composition associated with the germinated barley grains shifts considerably, not only quantitatively but also qualitatively (Petters *et al*

1988, Laitila *et al* 2007). Results of the study undertaken to examine the shifts in microbial community structure during malting (Chapter V) further confirmed these shifts and also revealed that malting reduces microbial diversity thus indicating the selection and dominance by process-dependant microorganisms.

These process selected microbes can either be beneficial or deleterious to malt quality such as those responsible for producing PYF causing factor/factors. The PYF responsible factor/factors are known to be considerably augmented during malting (Armstrong and Bendiak 2007, Axcell *et al* 1986, Axcell *et al* 2000, Blechová *et al* 2005, Griggs *et al* 2008, Sasaki *et al* 2008, van Nierop *et al* 2004, Yoshida *et al* 1979). If this is the case then the control of these microbes during malting might be an option for controlling or at least ameliorating the PYF problem. However, this will not to be an easy option until the identity of the causal agent/agents is unknown. To date no physical or chemical analysis methods have been developed to routinely detect the presence of the PYF factors in malt or barley. Consequently, the brewing industry relies on fermentation assays (Table 1.3, Chapter I) that are expensive, time consuming, and inconsistent (Lake and Speers 2008). Some success has been reported in the down sizing, and increasing the speed of these assays although real problems remain in the transferability and reproducibility of these assays between testing laboratories. The lack of consensus with respect to selecting a universal standard assay for PYF is a substantial problem, which makes comparison of research reports on PYF difficult. As was found in this study, some PYF +ve designated malt samples were suspected as PYF –ve or vice versa (Chapter IV). Despite the consensus of opinion being that PYF stems from microbial contamination of barley/malt, relatively little work has been reported on linking specific barley or malt microbial taxa with PYF malt (Table 1.4, Chapter I). Previous research investigations have primarily been directed towards identifying the causal wort PYF active components in PYF malt (Table1.2, Chapter I). However, these approaches have not been particularly successful over the past 40+ years in either identifying the causal PYF component/s or microbial organism/s.

In this thesis (Chapter IV) an attempt was made to characterise the responsible microbe/microbes (both bacteria and fungi) using DNA fingerprinting techniques. To my knowledge this is the first time such a study, using such a large number of PYF malt samples (41 geographically diverse) has been undertaken to identify the malt associated microbial taxon/taxa that predispose malt to the PYF problem. As shown in Figure 4.9 and Table 4.9 a definitive identification of PYF +ve and -ve malt samples by small scale fermentation tests presents real difficulties in identifying the causal microbes and eventually the causal factors. The relatively large and diverse number of samples used in this study has to a large extent overcome this difficulty and presented a viable alternative.

Visual observation and computation of average peak area of *Hae*III restriction enzyme for the 360 – 460 bp region of the fungal electropherograms indicated clear differences between PYF +ve and –ve malts. The PYF +ve malts tended to have greater number, as well as abundance of peaks in this region as compared to PYF –ve malts. ANOSIM of the TRFLP data for the *Hae*III enzyme showed clear differences in fungal community structures of PYF +ve and –ve malts (Table 4.3). Overall, the box plots of the 360 – 460 bp region of the electropherogram scores and peak areas showed that scores/peak areas were higher for PYF +ve than PYF –ve malts (Figure 4.9). In addition, the average relative abundance of the observed TRFs between the PYF +ve and –ve malts showed substantial differences in the population structure (Figure 4.3). Visual assessment of bacterial TRFLP electropherograms did not reveal obvious differences between PYF +ve and –ve malts (Figures 4.6 and 4.7). To my knowledge, this is the first study where bacterial communities have been studied to ascertain if they are associated with the occurrence of PYF. Thereby this study confirms that fungal taxa are the most likely cause of PYF as previously suggested by Lemos *et al* (2001) and van Nierop *et al* (2004). The malt origin was also found to have a significant effect on microbial community structure.

The presence of more than one peak in the 360 – 460 bp regions is suggestive of involvement of more than one fungal taxon in the development of the PYF problem. This is consistent with the conclusions of other investigations (Blechová

et al 2005, Sasaki *et al* 2008, van Nierop *et al* 2004, Yang *et al* 2007). Furthermore, the range of peak areas/visual score of electropherograms rather than a discrete value for PYF +ve malts and also the presence of TRF peaks although in less number and less abundance in PYF –ve malts suggest that PYF responsible microbes are also present in the PYF –ve malts but at lower levels. Similarly, the likely proportions of total microbial taxa that are associated with PYF are relatively small. This will impact on the ease of identifying the PYF causal taxa. The problem of PYF only appears to occur when the number of PYF associated taxa increase beyond a certain threshold, yet to be defined.

The TRFLP electropherograms for "secondary" type PYF +ve and –ve malts from one provider (Figure 4.7) showed peak areas for the 360-460 bp area that were relatively high and similar, but the areas >460 bp were greater in the secondary PYF+ve malts. This is suggestive of different or additional fungal taxa being responsible for the secondary type of PYF. However, only a limited number of defined secondary PYF+ve or –ve malt samples have been assessed. Further work is required with greater sample numbers to increase the confidence in the conclusions with respect to secondary PYF.

The diagnostic value of the 360 – 460 bp TRF region to discriminate between PYF +ve and –ve malts was compared directly with the small scale fermentation assay developed by Lake *et al* (2008). Of the three diagnostic statistics produced by the small scale fermentation test; 1. inflection point (M), 2. final wort gravity (^{o}Plato), and 3. turbidity (A_{600}); the turbidity (A_{600}) exhibited an almost perfect match with the TRFLP determination of PYF (Table 4.9) with 12 samples. That is the PYF +ve malt samples tended to have lower turbidity i.e. less number of yeast cells suspended in the medium (wort) indicating their sedimentation due to flocculation.

6.3 Future directions

Given the evidence that there is a link between PYF and fungal contamination, work will be ongoing to explore this association and further the understanding of this problem to eventually develop an effective solution. The TRFLP and cloning

and sequencing data will be mined to identify the causal PYF (for both primary and secondary) fungal taxa. Their identity will enable the design of specific PCR primer sets that will facilitate rapid and efficient identification of PYF +ve malts and potentially barleys. It is anticipated that this test will be based on specific sets of PCR primers to key PYF associated taxa that produce the 360-460 bp *Hae*III TRFLP fragments for primary PYF and the > 460 bp *Hae*III TRFLP fragments for secondary PYF. Further quantitation of these microbes is possible using various molecular techniques such as real time PCR, phylochips etc.

Validation of PYF identification by TRFLP both, for primary and secondary PYF associated fungal taxa using additional PYF sensitive brewer/s samples (PYF +ve and –ve both) will be undertaken. However, more interaction, collaboration and traceability between the malting and brewing industry, and academics are required to finally solve this longtime problem. Such a research perspective and understanding is likely to shed more light on the mechanism of PYF and also the understanding of yeast flocculation *per se*. Once the causal microbe/microbes are identified there is also an opportunity to isolate and identify the PYF responsible component/components and their mechanisms of action then can be studied.

Now it is established that geographical growing location of barley as well as the site of malt production, in addition to the specific malting protocols, determines the final microbial status of malt. These factors can be further explored to target specific problems or opportunities to improve malt quality by the malting and brewing industries rather than applying generalised solutions.

Recent advances in microbial technology like DNA fingerprinting techniques have greatly improved our ability to visualise and quantify microbial diversity in their natural environments. This technology has prompted a new era of microbial exploration. Information gained on the microbes, their growth, survival and death can then potentially be summarised in mathematical models for predictive microbiology. There are a whole range of mathematical equations and models that have been or are being constructed for major food borne pathogens and important spoilage organisms (McKellar and Lu 2004, McMeekin *et al* 1993, Peleg 2006),

but these have never been reported in the malting or brewing environment. Further, combined with knowledge of the intrinsic properties of barley grains and extrinsic factors such as barley growing, malting and brewing conditions these models may be used to predict the extent and probability of microbiological developments leading to fail safe to avoid undesirable problems such as gushing, mycotoxins, and PYF.

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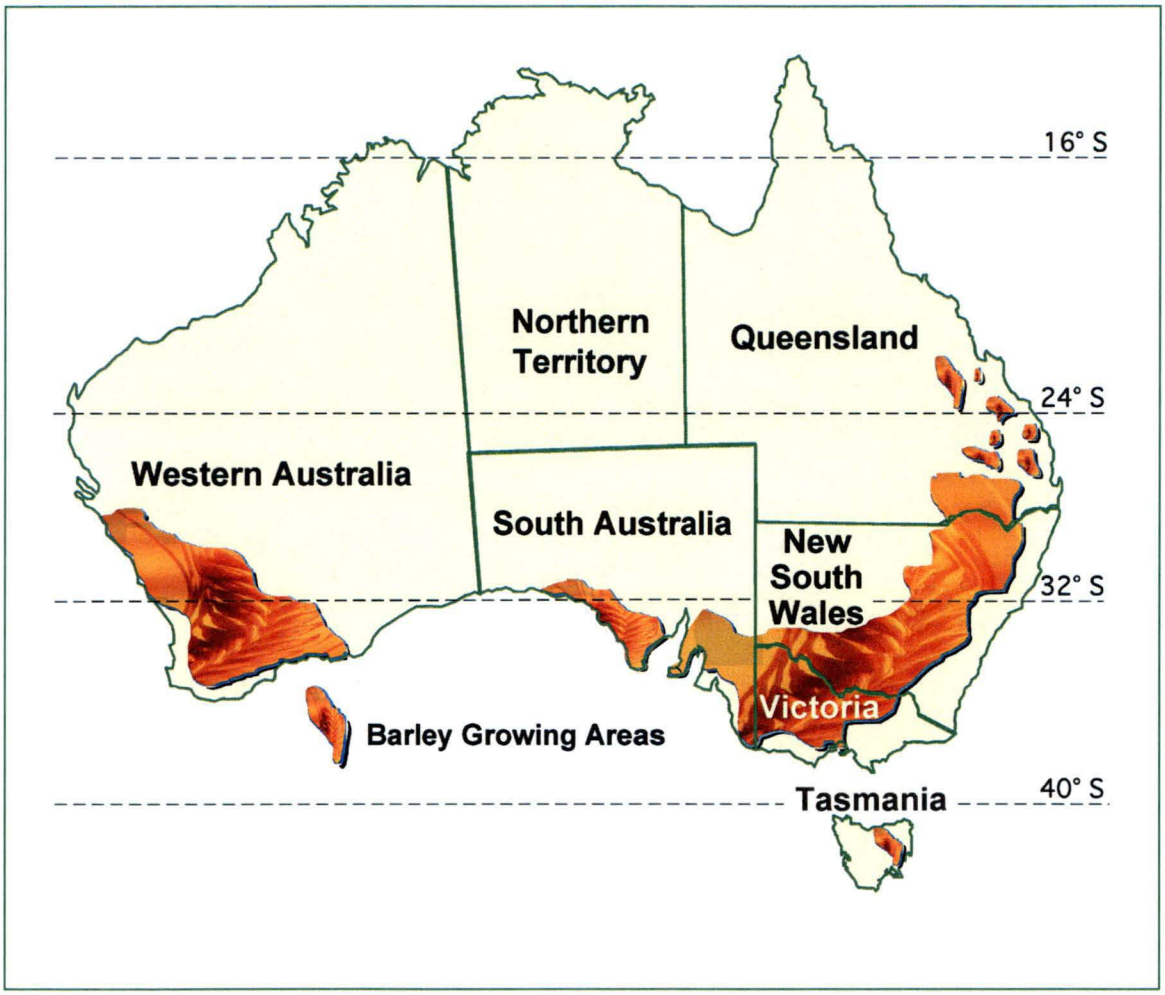
Appendix A

Major areas of barley production in Australia and locations from where barley and malt samples have been collected for studies in Chapters III and V



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